

Changes to the cytoskeleton and cell wall underlie invasive hyphal growth.

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ABSTRACT

Tip growth is a form of cellular expansion characteristic of fungal hyphae and some types of plant cells. Currently there is no unified model that satisfactorily describes this in hyphal species. Traditionally turgor has been considered an essential driving force behind cell expansion. In recent years this hypothesis has been challenged by evidence that in some species tip growth can occur despite the absence of measurable hydrostatic pressure. There are currently two contentious theories of hyphal extension. These are the turgor-driven model and the amoeboid-movement theory. Though the essential mechanism underlying cell growth differs between these theories, the actin cytoskeleton is considered important in both. It has been suggested that both the turgor-driven and amoeboid-like modes of growth could occur depending on whether the hyphae are growing invasively or non-invasively respectively (Money, 1990). It has also been proposed that both modes may occur within the same mycelium (Garrill, 2000). Two distinct patterns of actin have been identified in the hyphal tips of oomycetes. This has led to the hypothesis that two different mechanisms of apical extension may be employed by some hyphal organisms. During the course of this thesis, actin deplete zones have been observed in a significantly higher number of invasive compared to non-invasive hyphae of the oomycete *Achlya bisexualis*. Furthermore the difference between burst pressures was found to be lower in invasive hyphae compared to non-invasive hyphae suggestive of a weaker cell wall. A lack of significant difference in turgor pressures between the invasive and non-invasive hyphae of this organism suggests that the deplete zone and weaker wall plays a functional role in enabling hyphae to penetrate substrate. Fractal analysis of mycelial colonies shows that the variation in agar concentration and therefore substrate solidity has a significant effect on mycelial morphology. This is most likely due to an effect at the cellular level. The results of the experiments carried out during the course of this thesis provide the basis for future work towards elucidating the mechanisms of hyphal extension.

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Abbreviations and definitions

ABP	Actin binding protein
ADZ	Actin deplete zone
ADP	Adenosine diphosphate
ADP-Pi	Adenosine diphosphate and an inorganic phosphate
ATP	Adenosine triphosphate
D_{BS}	Border fractal
Ca^{2+}	Calcium ion
$\Delta\psi$	Change in osmotic potential (bars)
Δc	Change in solute concentration (mol L^{-1})
Da	Daltons
$^{\circ}\text{C}$	Degrees celsius
ε	Elastic wall modulus
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
F-actin	Filamentous actin
ADP-F-actin	Filamentous actin with an associated ADP
ADP-Pi-F-actin	Filamentous actin with an associated ADP-Pi
R	Gas constant ($8.23 \text{ J mol}^{-1}\text{K}^{-1}$)
G-actin	Globular actin
ATP-G-actin	Globular actin with an associated ATP
g l^{-1}	Grams per litre
Ψ_g	Gravimetric potential
Lp	Hydraulic conductivity
Mg^{2+}	Magnesium ion
D_{BM}	Mass fractal
MPa	Mega-pascal
μm	Micrometre
mm	Millimetre
mM	Millimoles
mOsmol/kg	Milliosmol per kilogram
M	Molar (moles per litre)
mol L^{-1}	Moles of dissolved solute per litre (osmolarity)
nM	Nanometer
Ψ_{π}^i	Osmotic potential of the cell

Ψ_{π}°	Osmotic potential of media
Pa	Pascal
PYG	Peptose-yeast-glucose
%	Percent
PIPES	Piperazine-N,N'-bis-[2-ethanesulfonic acid]
pH	Potential of hydrogen
Y_p	Pressure potential
Ψ_p	Pressure potential
Y_s	Solute potential
Ψ_s	Solute potential
\pm	Standard deviation
T	Temperature
v/v	Volume/volume
Y_w	Water potential
Ψ_w	Water potential
w/v	Weight/volume

CHAPTER 1

Introduction

Tip growth is a specialised form of cell expansion employed by fungal hyphae and some types of plant cells. It has traditionally been accepted that turgor pressure drives the extension of hyphae through the pressure exerted by the protoplast on the cell wall. Wall yielding occurs at the areas of directional expansion (as in Figure 1.1) allowing extension of the cell. There are three major models regarding the mechanism of wall extension and yielding.

The model proposed by Bartnicki-Garcia (1972) considers the increased plasticity of the wall at the extreme apex of the cell to be due to the excretion endonucleases which have been shown to weaken the bonds between the cell wall polymers (Cosgrove, 1987; 1993; 2000; Hill and Mullins, 1979, Money and Hill, 1997; Thomas and Mullins, 1967). The increased plasticity allows turgor to drive localised extension in this region. Wessels (1986) suggested that regionalised extension of the apical region of the wall is due to the active exocytosis of wall polymers. The polymers at the apex would initially exhibit only limited cross-linking resulting in a zone of high plasticity. This would allow turgor to force cell extension. Eventually the polymers would form more cross-links creating a more rigid subapical wall. The model proposed by Heath (1995) expounds the importance of the cytoskeleton in apical extension. Unlike the other models which consider the process of tip growth a balance between the pressure exerted by turgor and the localised extensibility of the cell wall, this model proposes that tip expansion is regulated by the cytoskeleton and in particular the peripheral network of the cytoskeletal protein actin (Pantaloni et al, 2001). Heath theorised that this network is attached to the plasmalemma and the cell wall by integrin-like linkages thus placing control in the cytoplasm, by way of normal intracellular regulatory systems (Kaminskyj and Heath, 1995, 1996). This theory does not explicitly require turgor for growth. It suggests that the cell may be restrained by the cytoskeleton under normal turgor conditions, but that extension is driven by the actin network under conditions of low cell turgor (Cooper, 1991)

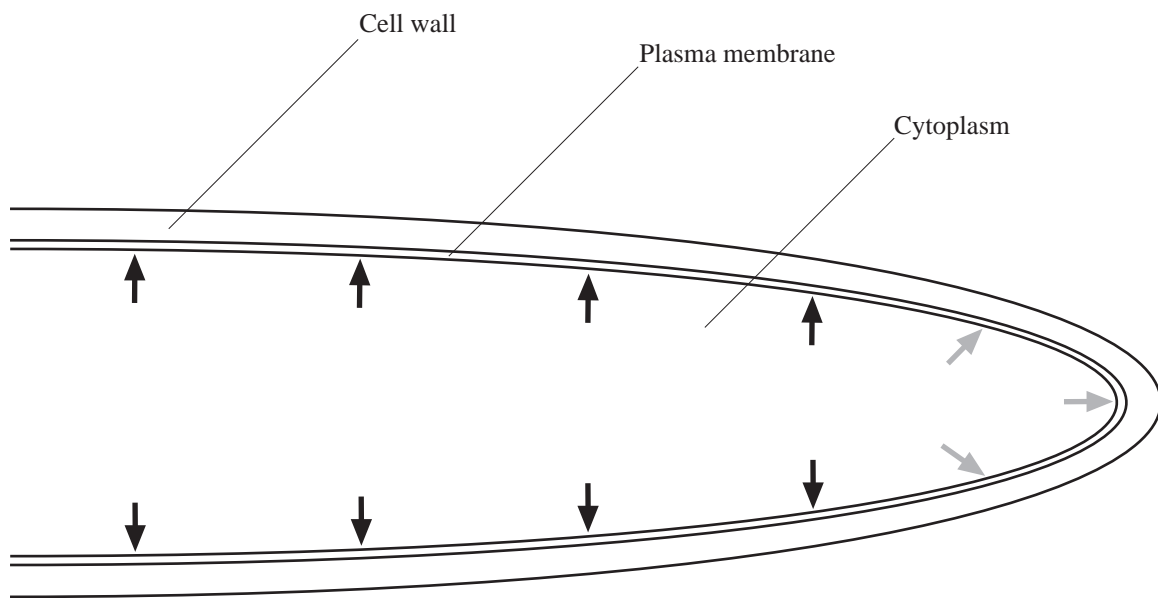


Figure 1.1 - A hyphal tip depicting the pressure applied to the cell wall by the turgid cytoplasm. The grey arrows indicate areas in which increased wall plasticity allows directional expansion of the cell. The black arrows indicate areas in which no expansion is occurring due to a decreased wall yielding ability. Adapted from Bartnicki-Garcia et al (2000).

The model proposed by Heath stems from the amoeboid movement theory which considers turgor less important in hyphal extension. It was first proposed by Reinhardt (1882), that although turgor was necessary to push the membrane against the cell wall, it was not necessary to drive extension. This hypothesis has been developed into the amoeboid-movement theory which likens hyphal extension to the locomotion of tube-dwelling amoebae. This involves the cytoplasm being protruded like a pseudopodium. The protrusion then adheres to the substrate by means of integrin-like proteins. A traction force is then exerted which moves the bulk of the cytoplasm towards the pseudopodium. The cell wall components are exocytosed and the wall is assembled on the surface of the cell (Harold et al, 1996).

The ability for fungi to invade or penetrate host tissue is important in pathogenesis (Money and Howard, 1996, Ravishankar et al, 2001). There is a lack of knowledge regarding fungal growth and this impedes our ability to control it. The predominating theories of growth are not mutually exclusive. It has been suggested that both non-turgor and turgor driven growth could occur within a single mycelium (Garrill, 2000). If this is correct, a better understanding of the modes of growth, the biochemical basis, and the mechanisms of switching from non-invasive to invasive growth, is required. Comparative analysis of the biochemistry and mycelial morphology of the different types of growth may provide important clues about how to stimulate, control, or prevent substrate invasion.

During the course of this thesis the role of the cytoskeletal protein, actin, in invasive and non-invasive growth is investigated. In addition to this biochemical analysis, the role of turgor pressure and cell wall strength in invasive and non-invasive hyphae and the effect of agar concentration on mycelial morphology are examined.

Actin

The cytoskeleton of eukaryotic cells is comprised of microfilaments, microtubules and intermediate filaments. The cytoskeleton provides the cell with the means to carry out a wide variety of functions required for growth, movement and reproduction (Alberts et al,

2002). Each component of the cytoskeleton is made up of proteins, arranged into filaments and each has unique properties and dynamics.

Microfilaments, or actin filaments assist in the maintenance of cell shape and provide support for the cell as well as aiding in cell locomotion (Wasteney and Galway, 2003). Microtubules are involved in intracellular transport and positioning of membrane bound organelles. Intermediate filaments give the cell mechanical support (Alberts et al, 2002). None of these components are functionally isolated. They are linked to cellular machinery and one another by accessory proteins which control everything from filament assembly and disassembly, to the translocation of organelles along the filaments.

Structure of microfilaments

Microfilaments are polymers of the protein actin. Actin was initially identified as a major constituent of muscle but was subsequently recognized as an important component of non-muscle cells in all eukaryotic organisms (Korn, 1982). Prokaryotic organisms do not contain actin but there is evidence for an ancestral form of actin present in the bacterial species *Bacillus* and some other species of non-spherical bacteria (Frankel, et al, 1990; Van Den Ent, 2001; Jones, 2001). This protein is called MreB and has an amino acid sequence homology to actin of 15%. Despite this, the size and shape of this protein strongly resembles actin (Van Den Ent, 2001).

Actin is a very highly conserved protein that can exist in two forms. Globular (G-) actin is the monomeric form of the protein and exists under conditions of low ionic strength. The actin protein has a molecular mass of nearly 43,000 Da and consists of a single polypeptide chain. Actin contains two domains which are called the “large” and “small” domains (this despite being very similar in size). There are four subdomains within the actin monomer (1-4 or IA, IB, IIA, IIB). Each subdomain contains a repeating motif comprising a multi-stranded β -sheet, a β -meander and a right handed $\beta\alpha\beta$ unit. 40% of the structure is comprised of α helices. There exists a cleft between the two domains in which there are high affinity binding sites for a nucleotide and a cation (Orlova et al, 1997). The binding of a nucleotide (usually ATP or ADP) in this cleft stabilizes G-actin

and prevents denaturation. The bound cation is usually Ca^{2+} or Mg^{2+} and assists binding of the nucleotide (Carlier et al, 1987; Selde et al, 1989). Consequentially the actin-binding affinities and rates of exchange of the nucleotide and cation are mutually interdependent. Both the nucleotide and the cation are freely exchangeable with exogenous nucleotides and cations. They are sensitive to changes in pH and ionic conditions because the affinity and kinetics of the divalent cation binding at the high affinity site are affected by concurrent cation binding at low affinity sites (Oda et al, 2001; Wang et al, 1989). G-actin also has four external divalent cation binding sites which, when occupied, cause the monomers to polymerize to form filamentous (F-) actin (Fechheimer and Zigmond, 1993). F-actin forms ultra structural networks that concentrate in the cortex of the cell and are associated with the plasma membrane.

Protofilaments are formed when monomeric actin associates in a head to tail or “pointed” to “barbed” orientation (Pollard, 1984). This reaction occurs when ionic strength is increased to that of physiological conditions (Oda et al, 2001). It is theorised that F-actin consists of two protofilaments arranged in a two start, right-handed, long pitch helix with structural polarity which is due to the organisation of the monomers within the polymer. Each monomer is associated with four surrounding monomers by an actin-actin binding site (Korn, 1982). These non-covalent associations between the monomers provide strength but allow for the flexibility observed *in vivo*. An alternate model has been proposed in which F-actin is considered a single start, left-handed, short pitch helix (Alberts et al, 2002; Holmes et al, 1990).

Monomeric actin associates to form F-actin by means of a condensation reaction (Pollard, 1984). The formation of F-actin under normal physiological conditions is characterised by a lag phase in which monomers associate to form unstable, dimeric nuclei (Sept et al, 1999). At this point the subunits are more likely to dissociate than to continue assembly. Once a stable trimer is formed there is a period of rapid elongation in which the nuclei grow into long filaments. This elongation process occurs at both ends of the filament but at different rates. The fast growing end is called the plus end and corresponds to the barbed end of the actin filament. The slow growing end is called the minus end and corresponds to the pointed end of the filament (Pollard, 1986). This difference in

association rates is due to a conformational change in each subunit as it enters the polymer (Alberts et al, 2002, Orlova and Egelman, 1995). Coupled to the polymerization of actin is the hydrolysis of the bound nucleotide (Kinosian et al, 1993). The nucleotide binding site on G-actin is almost always occupied by ATP *in vivo* (Carlier et al, 1987; Cooke, 1975). Once the ATP-G-actin has been incorporated onto the end of the actin filament forming ATP-F-actin, the nucleotide is hydrolysed to generate an intermediate form of actin filament containing ADP-Pi. This ADP-Pi-F-actin is thought to be a major intermediate in this process as the rate of release of the phosphate is much lower than the rate of nucleotide hydrolysis. Once the phosphate is released ADP-F-actin is formed (Cooke, 1975, Otterbein et al, 2001; Teubner and Wegner, 1998). This process is summarised in Figure 1.2.

The polymerisation of actin does not depend on ATP hydrolysis but the normal functioning of F-actin does (Korn et al, 1987). The elongation of the growing filament equilibrates when the number of monomers in solution reaches the critical concentration. At this critical concentration the rate of subunit addition equals the rate of subunit loss (Korn, 1982). Once the linear polymer of actin is formed, the exchange of monomers does not cease. The filament polymerises and depolymerises by the addition and removal of the actin subunits, which occurs at both ends of the filaments. The rate of monomer exchange is different for the pointed and barbed ends. The critical concentration for the barbed end is 12- to 15-fold lower than the pointed end under normal physiological conditions (Chen et al, 2000; Korn, 1982, Pardee et al, 1982; Wegner et al, 1983). This results in a biased rate of growth at the two ends of the filament as monomers tend to dissociate from the pointed end and associate at the barbed end (Schafer and Cooper, 1995). This process is called treadmilling.

Under *in vivo* conditions there are other proteins that are associated with both G- and F-actin. These are collectively known as actin binding proteins (ABPs) and many of them have regulatory roles in the assembly and disassembly of the microfilaments (Korn, 1982; Weber, 1999). ABPs have been divided into seven categories (Dos Remedios et al, 2003; Pollard and Cooper, 1986). These are defined by the functions of the proteins and include:

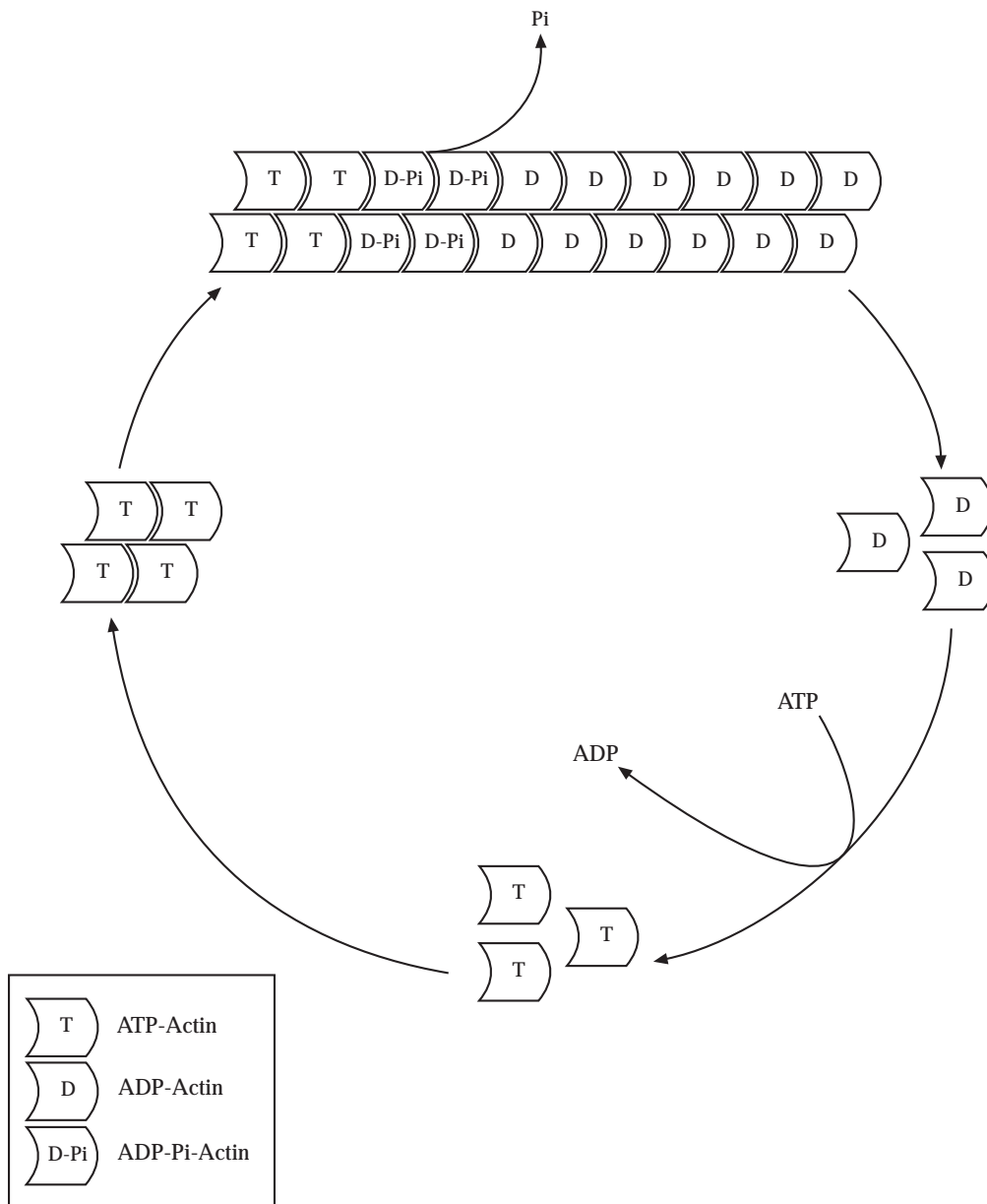


Figure 1.2 - The processes involved with the formation of microfilaments. Note the ATP caps are formed due to a lag in the nucleotide dephosphorylation subsequent to the incorporation of the nuclei onto the filament. Adapted from Chen et al (2000).

- Monomer binding proteins that associate with G-actin and prevent polymerisation.
- Filament depolymerising proteins that induce the conversion of F-actin to G-actin and speed up the rate of treadmilling.
- Capping proteins that bind to the pointed end of actin filaments and prevent the exchange of monomers.
- Filament severing proteins that bind and cut F-actin.
- Cross linking proteins that contain two actin binding sites and can therefore form bridges between two filaments. This assists in the formation of filament bundles and branch sites.
- F-actin stabilising proteins that prevent depolymerisation.
- Motor proteins that assist in the movement of cellular machinery and filaments relative to one another.

Proteins that associate with actin can be members of several of these categories and combined with the flexible, dynamic actin network can provide a vast array of functions necessary for cell growth, replication and survival (Chen et al, 2000).

Signalling to the cytoskeleton

There is an extensive and well documented connection between extracellular signals, membrane receptors, intracellular signals and the regulators of actin microfilament assembly (Alberts et al, 2002). These pathways have been shown to have effect right down to the level of gene expression. The signalling pathway from plasma membrane to effector molecule is a complex signalling network that involves many proteins and signalling factors (Heath, 2000a; Maniotis et al, 1997; Miyamoto et al, 1995). It is clear that signalling pathways converge on or diverge from individual proteins and

factors in a pathway. Such interactions arise from the existence and function of multiple signalling modules in the proteins involved in these pathways. There is a growing list of several thousand protein kinases and signalling factors that portend a complex and interwoven network of signalling interactions in cells (Dos Remedios et al, 2003).

One of the essential functions of the cytoskeleton is the ability to respond to cues from the environment (Heath, 2000a; Beck et al, 2001). In non-muscle cells, actin undergoes depolymerisation and repolymerisation in response to external and internal stimuli, and as needed for specific functions (Maniotis et al, 1997). External signals can affect the cytoskeleton through diverse routes and actin is linked to the plasma membrane in a variety of ways (Brakebusch and Fassler, 2003; Baluska et al, 2003). The simplest connections occur when actin filaments bind directly to proteins that span the lipid bilayer. The more common interaction is indirect. There are many proteins that act as links between membrane bound elements and the actin network. In this way signals from cell surface receptors can be transmitted to the cytoskeleton. Rho GTPases for example, play a central role in linking extracellular signals to the actin cytoskeleton as well as controlling cellular pathways that can activate transcription factors (Dos Remedios et al, 2003).

The functions of actin

Most eukaryotic non-muscle cells contain a cortical layer of actin filaments associated with the inner surface of the cell membrane (Alberts et al, 2002). This stratum provides the cell with mechanical support and assists in cellular movement. This movement is required for a variety of functions which includes extending surface areas, crawling, endo/exocytosis and cell shape alteration occurring during division. Not all of these functions are required for polarized tip growth but actin remains an important factor in this process. Significant roles associated with microfilaments include localisation of plasma membrane associated proteins, organelle movement and positioning, vesicle transport and cytoplasmic migration (Bachewich and Heath, 1997).

Actin in polarized, tip growing cells can form several different structures (De Ruijter et al, 2001; Geitmann and Emons, 2000, Vidali and Hepler, 2001). These include patches or plaques which are found throughout hyphae. Plaques are often associated with fibrillar networks of actin called arrays. The actin filaments in these arrays are orientated along the axis of the cell. It is thought that these arrays are central to the cytoskeletal network and enable the microfilaments to carry out many of the functions required for cell growth and development. There is a high degree of diversity between cell types regarding actin structure (Bachewich and Heath, 1997). This presumably is due to the different functions of the cytoskeleton within these cells. Hyphal cells, pollen tubes and root hairs have been compared as there is a high degree of similarity in their physiology and methods of extension (Anderhag, 2000; Baluska et al, 2001). As the cytoskeleton is important in tip growth, the role that it plays in these cells has been widely studied (Schafer et al, 1998). These studies are discussed further in Chapter 3.

Turgor

Water constitutes 80 – 90% of a cell and beyond its role in metabolism and nutrition, water influences the growth of plant and fungal cells through osmotic and hydrostatic pressure (Bray et al, 1991). Hydrostatic pressure is the force that is exerted on cell walls and membranes by aqueous solutions. Plants and fungal cells can generate large intracellular pressures that apply force to the cell wall. This is known as turgor and is brought about by a difference in water potential between the external and cellular environments.

Turgor is thought to be essential in many cellular and physiological processes such as cell enlargement, gas exchange in leaves, transport in the phloem, and various transport processes in membranes. As detailed earlier, the process of tip growth may be driven by the force exerted on the cell wall by the cytoplasm. Expansion of these tip growing cells is thought to occur at localised regions of the wall where interplay of turgor pressure and plastic deformation of the wall results in directional growth. However the exact role that pressure plays in this process remains somewhat equivocal partly due to the fact that few direct measurements of hyphal turgor have been made (Kaminskyj et al, 1992). Indirect methods can be employed but these can

be unreliable. Some evidence suggests that growth occurs in the absence of any measurable intracellular pressure (Harold et al, 1996, Money and Harold, 1993). As the function of turgor in hyphal cells is disputed, the alternative amoeboid-movement mechanism has been proposed (Burstrom, 1971). As detailed earlier this likens polarised growth to the movement of amoeboid-like organisms (Heath and Steinberg, 1999). Not all forms of growth can be explained by either hypothesis. Money (1999a) suggested that turgor pressure was required for invasive growth but not non-invasive growth. Garrill (2001) hypothesised that both modes of growth could occur in the same mycelium. In any study of turgor it is necessary to first consider the basics of cell water relations.

What is turgor pressure?

Turgor pressure is commonly defined as being the pressure exerted on a the cell by the turgid protoplasm which is bought about by the difference in the osmotic potentials of the protoplasm and the external environment. The chemical potential of water in a cell or its surroundings is described as “water potential”. Pure water has a water potential equal to zero. Addition of solutes lowers the water potential. When the water potential becomes higher, the hydrostatic pressure of a solution is increased. Differences in the water potentials drive the movement of the water causing it to flow from areas of higher water potentials to those of lower water potential. The contribution of solutes to water potential is called osmotic potential and can be measured. It should be noted that the term osmotic pressure is commonly used in the literature and is equal to osmotic potential but opposite (-) in sign. When the osmotic potential inside a cell is lower than that of the surroundings, water flows into the cell and turgor rises until the water potentials either side of the membrane are balanced. This is because the water flux into the cell can be counteracted by applying pressure to the inside of the cell. Osmotic pressure is the measurement of this force.

The term osmotic adjustment is used to describe the process by which a cell maintains the appropriate concentration of solutes to maintain water influx (Money, 1994). This is required because cells increase in volume as they grow which results in a drop in turgor. This term encompasses the continuous regulation of internal solute concentration during normal growth as well as specific responses to environmental

stress. Osmotic adjustment is a complicated process involving the synthesis or acquisition of compatible solutes, as well as the uptake and expulsion of solutes by the cell through both active and passive mechanisms.

Other factors contributing to water potential

Three major factors contribute to cell water potential (ψ_w). These are solutes, pressure, and gravity.

$$\psi_w = \psi_s + \psi_p + \psi_g \quad \text{Equation 1.1}$$

The term ψ_s is the solute potential or osmotic potential and represents the effect of dissolved solutes on water potential (ψ_w). Solutes lower the free energy of the water by diluting it. This is effectively an entropy effect in that the mixing of solutes and water increases disorder of the system, thereby lowering the free energy. The entropy effect of the dissolved solutes is revealed by other physical effects known as the colligative properties of solutions. Solutes reduce the vapour pressure, raise the boiling point, and lower the freezing point of solutions. The specific nature of the solute/s does not matter but the concentration of the total ions in solution does have an effect. For dilute solutions of non-dissociating substances, the osmotic potential of a solution can be calculated using the van't Hoff equation.

$$\psi_s = - RTc_s \quad \text{Equation 1.2}$$

In this R represents the gas constant which is given the value $8.32 \text{ J mol}^{-1}\text{K}^{-1}$. T is the absolute temperature in degrees Kelvin. The solute concentration of the solution is expressed as c_s and is otherwise known as osmolarity. The units for this are moles of total dissolved solutes per litre of water [mol L^{-1}]. For solutes that dissociate this is multiplied by two as there are effectively twice as many molecules of solute present. The minus sign indicates that dissolved solutes reduce the water potential of a solution. This equation is only suitable for “ideal” solutions at dilute concentration. In reality solutions often deviate from this ideal, but for the sake of argument all usage of this equation assumes situations are suitable for the application of this equation.

The hydrostatic pressure of the solution is sometimes denoted ψ_p (See Equation 1.1). Positive hydrostatic pressure raises the water potential and negative hydrostatic pressure reduces it. Hydrostatic pressure is measured as the deviation from ambient pressure so at a standard state, the ψ_p of water is zero. When referring to positive hydrostatic pressure within cells, ψ_p is usually called the turgor pressure. The effect of gravity is denoted by ψ_g (see Equation 1.1). This is dependant on the height of the potential water movement. The effect of gravity on ψ_g depends on the height of that water above the reference-state water, the density of water and the acceleration due to gravity. This gravitational component is often ignored, because when the vertical distances involved are small (such as those in a hyphae or any other type of cell) it is negligible. Collectively ψ_p and ψ_g can be called pressure potential as the medium surrounding the cell is almost always at atmospheric pressure and therefore can be considered a constant. This means that the water potential can be estimated using ψ_s and ψ_p alone and the equation can be simplified to:

$$Y_w = Y_s + Y_p \quad \text{Equation 1.3}$$

In this equation Y_w is equivalent to ψ_w , Y_s corresponds to ψ_s and Y_p is comparable to $\psi_p + \psi_g$, and is measured in pascals (Pa).

How are water relation parameters measured?

There are a limited number of techniques that can be used to study the water relations of individual cells (Money, 1990). One of the most commonly employed indirect techniques is the use of plasmolysed cells to estimate solute concentration in the cell, compared with that of the surrounding medium. With this technique the cells are exposed to a hypertonic solutions and the rate of shrinkage or swelling of the protoplasts are measured (Lew et al, submitted for publication). The gradients of osmotic pressure between the intracellular and extracellular environments drive water flow across the membrane (Jennings, 1995). From the rate of shrinking or swelling of a protoplast the hydraulic conductivity of the cell membrane (L_p) can be calculated. This technique is limited because the permeation of water across cell membranes is usually rapid resulting in an unstirred layer (Willmer and Beattie, 1978). This means that the osmotic concentration at the surface of the cell is not identical to that of the

bulk solution. Therefore these techniques tend to underestimate the hydraulic conductivity. Furthermore any movement of solutes into or out of the cell will bias any estimate of cell osmotic potential, compromising the results further. Another commonly employed indirect technique is psychrometry which is based on the fact that as vapour pressure is lowered, the water potential is reduced. This is one of the colligative properties of a solution. Psychrometers are used to measure the vapour pressure and therefore the water potential of cytoplasm through estimations of evaporative cooling. This technique however does not preserve the sample for any further analysis.

Direct measurements, while technically demanding, provide a far more accurate assessment of the water relation parameters of individual cells. The use of a cell pressure probe largely circumvents problems associated with indirect techniques, while still providing the means to estimate such factors as cell turgor pressure, hydraulic conductivity (L_p) and elastic wall modulus (ϵ) which can potentially provide important clues as to the mechanisms involved in cellular processes such as polarised cellular extension (Cosgrove et al, 1987, Steudle, 1998, 2003).

The function of pressure in cells

The cells of plants and fungi have a cell wall. As turgor develops the cytoplasm swells pressing the plasma membrane against the cell wall. The mechanical strength of the wall prevents the cell from bursting under normal physiological conditions (Cosgrove, 1987). The wall is flexible and polymeric yet is strong enough to constrain the cytoplasm (Bartnicki-Garcia et al, 2000). In order for cells to expand, the expansion of both the protoplast and the cell wall must occur. Upon movement of water into the cell, the wall stretches and due to its biophysical properties, elastic energy is stored in the polymers. This energy compresses the protoplast, increasing the pressure and therefore the water potential of the cytoplasm. When cell water potential reaches that of the external milieu, the flux of water into the cell ceases. In growing cells this equilibrium is never reached, as the wall at the apex relaxes, decreasing the turgor pressure of the cell. The subsequent influx of water into the cell results in expansion of the cell, again increasing the elastic energy of the cell wall (Eamus and Jennings, 1986b). In reality these processes do not occur in a stepwise

manner but simultaneously, resulting in cell growth rate and turgor remaining relatively steady.

There are several theories regarding the mechanisms of cell wall relaxation. The first considers wall stress relaxation to be a viscoelastic process. This term refers to the mechanical properties displayed by the cell wall, in that it appears to be viscous as well as retaining some elastic properties in response to stress. In many cells viscoelastic extension is driven by the turgid cytoplasm, and wall extension is considered a passive slippage of the polymers present in the wall matrix. In some situations this slippage is thought to be mediated by the cleavage of load-bearing cross-linkages between polymers. This is called a chemorheological process. When wall expansion is mediated by chemorheological processes then there will be little correlation between the growth behaviour of a cell and the viscoelastic properties of its cell wall. Situations such as this have been documented but there are also studies that correlate the viscoelasticity of the cell wall and growth rate. The alternative and more widely accepted hypothesis to that of passive viscoelastic extension is that a chemorheological process involving the action of wall loosening enzymes on the wall polymers allows turgor driven extension to occur. In some specific types of cells, enzymes have been shown to be secreted at those parts of the wall at which extension or expansion is occurring. For example, autolysins are cell membrane-associated wall-lysing enzymes that are responsible for keeping the wall at the hyphal apex soft and extensible. They act on the bonds between wall polymers, effectively solubilising them and allowing the deposition and incorporation of new molecules into the matrix (Cosgrove, 1993, 2000). Similar mechanisms have been proposed for the growth of bacterial and plant cells. The cell wall of plant cells which can be likened to that of that of oomycetes, consist of a hydrophilic matrix of hemicelluloses and pectins with an embedded polymeric network of crystalline cellulose microfibrils. The cell wall of fungi has a similar structure but contains lignin rather than cellulose.

Mycelial morphology

In studying the mechanisms of hyphal growth it should be kept in mind that hyphal organisms exist for the most part not as single hyphae but as a mass of interconnected branching hyphae or mycelium. A comprehensive investigation of growth should

thus scale up from the hyphae to the mycelium and should be mindful that in nature the component hyphae of a single mycelium may exist in different environments. It is well established that environmental conditions can affect the growth of organisms. Variations in behaviour, reproduction, growth and morphology can be observed in species occurring in distinct environments. Some of these variations can be developmental whereas others are in temporal flux, subject to dynamic changes in response to changing environmental conditions. Responses such as these can occur in reaction to a vast number of variables, such as time, inter- or intraspecific competition, water potential, temperature, and nutrient availability. Many such variables can affect the morphology of mycelial colonies. These factors can be subject to temporal and spatial variation. Some environmental conditions are influenced by the organism itself, for instance water potential and pH. The effects of the physical environment are interactive making *in situ* analysis very difficult. As a consequence, little is known about the effect of environmental heterogeneity on mycelial development and morphology.

The quantitative analysis of complex three dimensional structures is relatively difficult. Currently the most effective method for studying such systems is the technique called fractal geometry. As hyphal colonies are approximately fractal, fractal geometry can be applied to them (Boddy et al, 1999; Bolton and Boddy, 1993; Donnelly et al, 1995). This describes the morphological characteristics of a colony in numerical terms. Fractal geometry has been used to provide evidence for different growth strategies employed in varying environments. This is typically achieved through image capture and analysis (Donnelly et al, 1995; Hitchcock et al, 1996). Mass and border fractals are the two measurements typically made of fungal systems. This requires the analysis of the space filling capacity within the system and at the mycelial margins respectively.

Mycelial growth strategies

Two types of growth are thought to be utilised by hyphal organisms. These two forms are known as the 'phalanx' growth strategy and the 'guerrilla' growth strategy (Lundy et al, 2001). The former describes a pattern of growth which displays tightly aggregated rosettes of mycelia. These are relatively slow growing and typically

develop in rich media substrates. The latter involves the development of loosely aggregated, fast growing branches within mycelia. The guerrilla growth strategy occurs when the organism is growing on nutrient-scarce substrates and is hypothesised to be a foraging mechanism. Broad-fronted, slow growing mycelia exhibiting phalangeal growth are thought to utilize diverse and locally abundant resources. These morphological and physiological variations are thus related to the nutrient and resource requirements of the organism.

Fungi can be classified as either unit restricted or non-unit restricted. This refers to the ability of the organism to utilize different substrates for the acquisition of nutrients. Unit-restricted fungi can only grow on specific media or substrates and colonize new environs through spore dispersal. Non unit-restricted fungi can extend to new reservoirs of resources through growth of mycelia as well as by spore dispersal. Unit-restricted systems tend to produce mycelia displaying a phalangeal growth pattern, whereas non unit-restricted fungi have a propensity to utilise the guerrilla growth strategy. Significantly, differences in colony morphology also correlate with extension rate of the hyphae within the mycelium. Systems that are more phalangeal generally extend faster than those exhibiting a more guerrilla-like growth pattern (Donnelly et al, 1995), though this is also related to resource availability. The contrasting strategies as detailed above were first employed to describe the growth patterns in plants (Schmid and Harper, 1985), though subsequently they have been widely used to describe mycelial systems.

By investigating the differences between invasive and non-invasive hyphae, a better understanding of the mechanisms involved may be achieved. Analysis of the cytoskeleton, cell pressure and mycelial morphology were carried out during the course of this thesis, the results of which are detailed in the following chapters.

CHAPTER 2

Materials and Methods

Stock Cultures

Achlya bisexualis

Stock cultures of *Achlya bisexualis* Coker (a female strain isolated in New Zealand from *Xenopus laevis* dung, available from the University of Canterbury culture collection) were maintained at 25°C on peptone-yeast-glucose media containing (in g l⁻¹); glucose 3 (BDH, UK), bactopectone 1.25 (Gibco BRL, UK), yeast extract 1.25 (Gibco BRL, UK), and bacteriological agar 20 (Oxoid, UK), made up with distilled water. The organism was sub-cultured using a 5 mm diameter cork-borer to remove agar plugs from the growing edge of a colony on a stock plate.

Neurospora crassa

Stock cultures of *Neurospora crassa* type A (C212) (University of Canterbury culture collection) were maintained at 20°C on peptone-yeast-glucose media containing (in g l⁻¹); glucose 3 (BDH, UK), bactopectone 1.25 (Gibco BRL, UK), yeast extract 1.25 (Gibco, BRL, UK), and bacteriological agar 20 (Oxoid, UK), made up with distilled water. The organism was sub-cultured by using a 5 mm diameter cork-borer to remove agar plugs from the growing edge of a colony on a stock plate.

Mortierella wolfii

Stock cultures of *Mortierella wolfii* CBS 611.70 (obtained from Centraalbureau voor Schimmelcultures, Institute of the Royal Netherlands Academy of Arts and Sciences) were maintained at 40°C on malt extract media containing (in g l⁻¹); Malt extract 20 (Oxoid, UK), and bacteriological agar 20 (Oxoid, UK), made up with distilled water.

The organism was sub-cultured by using a 5 mm diameter cork-borer to remove agar plugs from the growing edge of a colony on a stock plate.

Experimental Cultures

***Achlya bisexualis* cellophane plates**

Cellophane plates were constructed by placing a 50 mm X 60 mm rectangle of colourless cellophane (Hallmark brand) on a peptose-yeast-glucose agar plate. Prior to use the cellophane was boiled for 10 minutes in distilled water to remove any manufacturing residues. This process was repeated three times prior to autoclaving. After placement on a peptose-yeast-glucose plate, the cellophane was inoculated with *Achlya bisexualis*. The inoculum was obtained by using a 5 mm diameter cork-borer to remove an agar plug from the growing edge of a colony on a stock plate. The plates were incubated at 25°C for 48 hours.

***Achlya bisexualis* overlay plates**

Overlay plates were constructed by placing a 50 mm X 60 mm rectangle of cellophane (Hallmark brand) on a peptose-yeast-glucose agar plate. The cellophane was prepared as per the protocol detailed above. Autoclaved 1% or 4% low melting point (LMP) agarose (Gibco BRL, UK) (3 ml – 6 ml) was then poured over the cellophane. Once set, the LMP agarose was inoculated with *Achlya bisexualis* and the plate was incubated at 25°C for 48 hours. The inoculum was obtained using a 5 mm diameter cork-borer to remove an agar plug from the growing edge of a colony on a stock plate. The overlay plates were incubated at 25°C for 48 hours.

***Achlya bisexualis* fractal geometry plates**

One percent, ten percent and one hundred percent peptose-yeast-glucose plates were made up containing the following concentrations of agar; 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8% (w/v). These correspond to 10, 20, 30, 40, 50, 60, 70 and 80 g l⁻¹ bacteriological agar (Oxoid, UK) respectively. The 1% peptose-yeast-glucose media

contained (in g l⁻¹); glucose 0.03 (BDH, UK), bactopectone 0.0125 (Gibco BRL, UK), yeast extract 0.0125 (Gibco BRL, UK). The 10% peptose-yeast-glucose media contained in g l⁻¹; glucose 0.3, bactopectone 0.125, yeast extract 0.125. The 100% peptose-yeast-glucose media contained (in g l⁻¹); glucose 3, bactopectone 1.25, yeast extract 1.25. All media were made up with distilled water. The plates were inoculated with *Achlya bisexualis*. The inoculum was obtained using a 5 mm diameter cork-borer to remove an agar plug from the growing edge of a colony on a stock plate. The plates were incubated at 25°C for 48 hours.

***Neurospora crassa* fractal geometry plates**

Peptose-yeast-glucose plates were made up containing in (g l⁻¹); glucose 3 (BDH, UK), bactopectone 1.25 (Gibco BRL, UK), yeast extract 1.25 (Gibco BRL, UK) and the following concentrations of agar; 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8% (w/v) again corresponding to 10, 20, 30, 40, 50, 60, 70 and 80 g l⁻¹ bacteriological agar (Oxoid, UK) respectively. All media were made up with distilled water. The plates were inoculated with *Neurospora crassa*. The inoculum was obtained using a 5 mm diameter cork-borer to remove an agar plug from the growing edge of a colony on a stock plate. The plates were incubated at 20° for 24 hours.

***Mortierella wolfii* fractal geometry plates**

One percent, ten percent and one hundred percent (w/v) malt extract agar plates were made up containing the following concentrations of agar; 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8% (w/v). These correspond to 10, 20, 30, 40, 50, 60, 70 and 80 g l⁻¹ bacteriological agar (Oxoid, UK) respectively. The 1% malt extract agar contained (in g l⁻¹); Malt extract 0.2 (Oxoid, UK). The 10% malt extract agar contained (in g l⁻¹); Malt extract 2.0. The 100% malt extract agar contained (in g l⁻¹); Malt extract 20. All media were made up with distilled water. The plates were inoculated with *Mortierella wolfii*. The inoculum was obtained using a 5 mm diameter cork-borer to remove an agar plug from the growing edge of a colony on a stock plate. The plates were incubated at 40°C for 24 hours.

***Achlya bisexualis* Gel-gro fractal geometry**

One percent (w/v) peptose-yeast-glucose agar plates were made up containing (in g l⁻¹); glucose 0.03 (BDH, UK), bactopectone 0.0125 (Gibco BRL, UK), yeast extract 0.0125 (Gibco BRL, UK) and 1%, 2%, 3%, 4% and 5% (w/v) corresponding to 10, 20, 30, 40, 50 g l⁻¹ Gel-gro agar substitute (ICN Biomedicals, Inc., USA) respectively. All media were made up with distilled water. The plates were inoculated with *Achlya bisexualis*. The inoculum was obtained using a 5 mm diameter cork-borer to remove an agar plug from the growing edge of a colony on a stock plate. The plates were incubated at 25°C for 48 hours.

Fractal geometry

Image capture and processing

Mycelia were digitally imaged using a Microtek Scanmaker V6USL Scanner (Microtek Lab Incorporated). The images were processed using Adobe Photoshop (Adobe Systems) software prior to fractal analysis. Processing was carried out as per Donnelley et al (1995) and involved the conversion of the pixels in the image to either black or white. Each pixel was assessed as 0 (black) or 255 (white) in relation to its shade on a continual grey scale of 0 to 255 thus reducing the image to binary data. The value that differentiated between black and white was termed the threshold and was set to a standard level for each species. Background interference was removed and then the image was analysed using fractal analyses.

Fractal analysis

Fractal analysis was carried out using ImageJ software (available online in the public domain at <ftp://rsbweb.nih.gov/pub/image-J/>). Mycelial area was calculated by counting the total number of black pixels in each image and multiplying by the area represented by each pixel. As a comparative study of area was being made, the area of the inoculation plug was not subtracted as this was constant across all treatments. ImageJ was used to calculate the mass fractal of each image by the box counting

method. This is described further in the introduction to Chapter 5. The border fractal images were generated by using the ImageJ outline function which removes all black pixels except those forming the very edge of the mycelia. The box count method was again employed to calculate the border fractal value.

Statistical analyses

Statistical analyses were carried out using Statistix 7 software package (Analytical Software).

Fixation and staining of non-invasive hyphae

Two methods were used to fix non-invasive hyphae:

A rectangle of *Achlya bisexualis* mycelia was cut using a sterile scalpel blade and removed from the margin of a culture growing on a cellophane plate. The hyphae were removed from the cellophane and left for an hour in peptose-yeast-glucose broth to allow them to resume growth. The hyphae were then fixed for 30 minutes using a solution containing (v/v) 0.5% methylgloxal (Sigma-Aldrich Co., USA), 4% formaldehyde (Sigma Chemical Co., USA) or 4% paraformaldehyde (ProSciTech, Australia), 50 mM PIPES pH 6.8 (Sigma-Aldrich Co. USA) and 5 mM EGTA (Sigma-Aldrich Co., USA), made up with distilled water. In initial experiments various concentrations of PIPES (0 mM, 50 mM, 75 mM and 100 mM) and EGTA (0 mM, 1 mM, 5 mM and 10 mM) were tested to determine the optimum combination of concentrations for fixation. Concentrations of 50 mM PIPES and 5 mM EGTA, which were found to give the best preservation of hyphae, were used for subsequent experiments. After fixation the hyphae were washed for 15 minutes in 50 mM PIPES solution. This was repeated twice. The hyphae were then stained for 30 minutes with Alexa Phalloidin (stock obtained from Molecular Probes and diluted 1:4 with 50 mM PIPES). During staining the sample was kept in the dark to prevent photo bleaching. After staining the hyphae were washed for 15 minutes in 50 mM PIPES. The rinsing was repeated twice. After the second wash an anti-fading agent (*p*-phenylenediamine

(Sigma-Aldrich Co. USA) made up to 0.1% in distilled water) was added to the sample. The hyphae were then examined using a MRC1024 confocal microscope (Bio-Rad, Canada) and an Olympus IX70 Epifluorescent microscope with an Olympus LCPlanF1 20X/0.4, an Olympus UApo/340 40X/1,35 Oil iris ∞ /0,17 objective or an Olympus UPlanApo 100X/1,35 Oil iris ∞ /0,17 objective. Some imaging was carried out on an Olympus BH2-RFCA Epifluorescent microscope with an Olympus DPlanApo10UV 0.40 160/0.17 (101326) objective, an Olympus DPlanApo40UV 1.00oil 160/- (107130) objective or an Olympus DPlanApo100UV 1.30 oil 160/0.17 (101076) objective.

For some experiments, an alternative technique for fixing and staining was used. The hyphae were prepared as described above. These were then fixed for 45 minutes in solution containing (v/v) 0.5% methylgloxal, 4% paraformaldehyde and 50 mM PIPES pH 6.8, made up with nanopure water. There was no variation in the type of formaldehyde used and no EGTA was added. After fixing the sample was rinsed in 50 mM PIPES for five minutes with shaking. This step was repeated five times. The hyphae were stained for 45 minutes with Alexa Phalloidin (stock obtained from Molecular Probes and diluted 1:4 with 50 mM PIPES). After staining the sample was rinsed in 50 mM PIPES for 15 minutes. The rinsing was repeated three times. After the second wash an anti-fading agent (*p*-phenylenediamine made up to 0.1% in distilled water) was added to the sample. The hyphae were then examined using the microscopes as detailed above.

Fixation and staining of invasive hyphae

Two methods were used to fix invasive hyphae:

A rectangle of *Achlya bisexualis* mycelia was cut using a sterile scalpel blade and removed from the margin of a culture growing on an overlay plate. The hyphae in low melting point agar were removed from the cellophane and left for an hour in peptose-yeast-glucose broth to resume growth. The hyphae were then fixed for 1 hour in solution containing (v/v) 0.5% methylgloxal (Sigma-Aldrich Co., USA), 4% formaldehyde (Sigma Chemical Co., USA) or 4% paraformaldehyde (ProSciTech,

Australia), 50 mM PIPES pH 6.8 (Sigma-Aldrich Co. USA) and 5 mM EGTA (Sigma-Aldrich Co., USA), made up with distilled water. Again, various concentrations of PIPES (0 mM, 50 mM, 75 mM and 100 mM) and EGTA (0 mM, 1 mM, 5 mM and 10 mM) were tested to determine the optimum combination of concentrations. Again 50 mM PIPES and 5 mM EGTA gave the best preservation and were used for subsequent experiments. After fixation the hyphae were washed for 30 minutes in 50 mM PIPES solution. This was repeated twice. The hyphae were then stained for 1 hour with Alexa Phalloidin (stock obtained from Molecular Probes and diluted 1:4 with 50 mM PIPES). During staining the sample was kept in the dark to prevent photo bleaching. After staining the hyphae were washed for 30 minutes in 50 mM PIPES. This was repeated twice. After the second wash an anti-fading agent (*p*-phenylenediamine made up to 0.1% in distilled water) was added to the sample. The hyphae were then examined using the microscopes as detailed above.

The alternative technique of fixing and staining involved hyphae being removed from a cellophane plate and placed in a well slide. The hyphae were overlaid with cooled 2% low melting point agarose. The sample was then covered with peptose-yeast-glucose broth and left for an hour to resume growth. The hyphae were fixed for 45 minutes in a solution containing (v/v) 0.5% methylgloxal, 4% paraformaldehyde and 50 mM PIPES pH 6.8, made up with nanopure water. There was no variation in the type of formaldehyde used and no EGTA was added. After fixing the sample was rinsed in 50 mM PIPES for five minutes with shaking. This step was repeated five times. The hyphae were stained for 45 minutes with Alexa Phalloidin (stock obtained from Molecular Probes and diluted 1:4 with 50 mM PIPES). The sample was then rinsed in 50 mM PIPES for 15 minutes. The rinsing was repeated three times. After the second wash an anti-fading agent (*p*-phenylenediamine made up to 0.1% in distilled water) was added to the sample. The hyphae were then examined using the microscopes as detailed above.

Pressure analysis

Pressure probe

Direct pressure measurements were made using a pressure probe designed and built by Professor Steve Tyerman, University of Adelaide, Australia. The pressure transducer was capable of measuring a maximum of 20 bars. For experimental purposes the pressure probe and attached micropipette were filled with low viscosity silicon oil (WACKER AS4, Wacker-Chemie, Germany). Borosilicate glass capillaries (Harvard Apparatus Ltd., U.K.) were used to make the micropipettes. These had an external diameter of 1.2 mm and an internal diameter of 0.69 mm. The pipettes were pulled on a Narashige PC-10 pipette puller (Narashige Co. Ltd., Japan) on a one step setting at a heater level of 60. The pressure probe was attached to micromanipulators (Lang GMBH & Co. K6, Type STM3) which were in turn attached to a Zeiss IM35 microscope with a Zeiss F-LD20/O,25 Phase objective. The turgor pressures of *Achlya bisexualis* invasive hyphae (from an overlay plate) and non-invasive hyphae (from a cellophane plate) were measured. These measurements were obtained by puncturing a hypha with the micropipette no more than approximately 300 µm from the apex of the cell. Upon entry of the micropipette into the cell, the oil-cytoplasm interface moved into the pipette. To measure the hydrostatic pressure of the cell the meniscus was moved to the point at which the micropipette entered the cell wall. This had to be repeated at least twice (and in most cases at least 4 to 5 times) for any one cell in order for the measurements to be used as results. The pressure readings were printed out using a Smiths RE511.20 Potentiometric recorder.

Burst pressures

To measure the burst pressure of the hyphae of *Achlya bisexualis* a pressure probe was used as detailed above. At least three measurements of the initial turgor were made. Once these recordings had been completed, oil was injected into the cell. The hyphae were monitored and the pressure and position at which the burst occurred was recorded.

Osmotic treatments

To assess the ability of hyphae of *Achlya bisexualis* to turgor regulate, a pressure probe was used as detailed above. At least three measurements of the initial turgor were made. Once these recordings had been completed the hyphae were exposed to hyperosmotic or hypoosmotic conditions. For hyperosmotic treatments, 2.5 ml PYG (containing (in g l⁻¹); glucose 0.03 (BDH, UK), bactopectone 0.0125 (Gibco BRL, UK), yeast extract 0.0125 (Gibco BRL, UK)) was supplemented with sucrose (BDH, UK) to give a solution with an osmolarity of 1244 mOsmol/kg. This was added to the 10 ml of PYG which surrounded the impaled hyphae. This PYG solution had an osmolarity of 43 mOsmol/kg. Upon the addition of the PYG-sucrose solution this increased to 283 mOsmol/kg. The change in osmolarity experienced by the hyphae was 240 mOsmol/kg or equivalent to a pressure change of 5.85 bars as calculated using the equation:

$$\Delta\psi = \Delta cRT \quad \text{Equation 2.1}$$

Where $\Delta\psi$ denotes the change in the osmotic potential (in bars), Δc denotes the change in solute concentration of the solution expressed as osmolarity (moles of total dissolved solutes per litre of water [mol L⁻¹]), R denotes the gas constant (8.32 J mol⁻¹ K⁻¹), and T denotes the absolute temperature (in degrees Kelvin). Alternatively 2.5 ml of BS containing 1000 mM sucrose (osmolarity 1427 mOsmol/kg) was added to the 10 ml of BS surrounding the hyphae (final osmolarity of 441 mOsmol/kg). The resulting osmolarity change was 246 mOsmol/kg, equivalent to an osmotic potential change of 6.0 bars, as calculated using Equation 2.1. The addition of the sucrose solution to the PYG or BS solution was carried out without stirring in order to minimise vibration and damage to the impaled hypha.

Incipient plasmolysis

Rectangles of *Achlya bisexualis* hyphae were cut using a sterile scalpel blade and removed from the margins of cultures growing on overlay and cellophane plates. The hyphae were separated from the cellophane and left for an hour in peptose-yeast-glucose broth to resume growth. The nutrient broth was then removed and replaced

with solution made up from peptose-yeast-glucose broth and supplemented with; either 0 M, 0.1 M, 0.25 M, 0.5 M, 0.75 M or 1.0 M sorbitol (Sigma Chemical Co., USA). The samples were left to plasmolyse for 30 minutes. Fifty hyphae in each sample were then assessed for plasmolysis using a Zeiss IM35 microscope with a Zeiss F-LD20/O,25 Phase objective. From the results a standard curve was constructed using SigmaPlot 2000 and fitted using a logistic regression analysis of the form:

$$y = \frac{a}{1 + \left(\frac{X}{X_o} \right)^b} \quad \text{Equation 2.2}$$

The concentration of sorbitol that caused 50 % of the hyphae to plasmolyse was calculated by the rearrangement of Equation 2.2. As described by Money (1990), hyphae will decrease in volume as they approach incipient plasmolysis, thus the protoplasm is much more concentrated than when the cells are at full turgor. To estimate the internal osmotic potential there needs to be a correction factor that compensates for this, otherwise this value would be underestimated. Hyphae of *A. bisexualis* have been found to decrease to a volume of 63% of that of cells at full turgor. (Money, 1990), so in these experiments the critical solute concentration has been multiplied by 0.63 to give the internal osmotic potential. Turgor was then calculated as the difference between the osmotic potential of normal PYG media and the calculated internal osmotic potentials as per the following equation:

$$\psi_p = \psi_{\pi}^o - \psi_{\pi}^i \quad \text{Equation 2.3}$$

Where ψ_p denotes turgor pressure, ψ_{π}^o denotes the osmotic potential of the media and ψ_{π}^i denotes the osmotic potential of the internal environment. Unsupplemented PYG media has an osmolarity of 43 mOsmol kg⁻¹ as measured with a Wescor vapour pressure osmometer (Model 5100C). This is equivalent to 1.06 bars. The conversion to bars was carried out using the van't Hoff equation:

$$\psi_s = - RTc_s \quad \text{Equation 2.4}$$

Where ψ_s denotes the osmotic potential (in bars), R denotes the gas constant ($8.32 \text{ J mol}^{-1} \text{ K}^{-1}$), T denotes the absolute temperature (in degrees Kelvin) and c_s denotes the solute concentration of the solution, expressed as osmolarity (moles of total dissolved solutes per litre of water [mol L^{-1}]). The minus sign indicates that dissolved solutes reduce the water potential of a solution.

CHAPTER 3

The cytoskeleton

The key processes in hyphal elongation are thought to occur at the extreme apex of the cell. Actin is a prominent feature within these tips suggesting it may play an important role in this process (Heath, 1990; Geitmann and Emons, 2000; Heath et al, 2003; Heath 2000b, Roberson, 1992). Indeed it has been suggested that actin is important in all tip growing cells (Gibbon et al, 1999; Gupta and Heath, 1997; Habel et al, 2003; Picton and Steer, 1982; Vidali and Hepler, 2001). There has been a large quantity of data published in support of this hypothesis and several models have been developed that involve actin playing an essential role in tip growth. Models regarding hyphal extension have been based on the additional premise that turgor is important. In recent years it has become evident that turgor may not be as important in cellular extension as previously hypothesised (Heath and Steinberg, 1999; Money and Harold, 1993). Throughout this, the importance of the actin cytoskeleton has been maintained (Heath et al, 2000; Ketelaar et al, 2003, Virag and Griffiths, 2004). The actual role of the actin cytoskeleton within growing tips of hyphae has been a matter of some debate. This matter arises from the different models of hyphal extension and from the fact that several different cytoskeletal structures have been observed in hyphae. In oomycetes such as *Saprolegnia* (Heath, 1987, 1988; Jackson and Heath, 1989) and *Achlya* (Yu et al, 2004), actin has been shown to form an apical cap of densely packed filaments with a sub-apical arrangement of plaques and fibrils (Heath, 1987, 2000b).

It has been suggested that the actin cap is the structure through which cellular polarity and hyphal shape is maintained and the means by which cellular apparatus is moved to and from the hyphal tip (Heath and Skalamera, 2001; Heath, 1990; Roberson, 1992). The network of microfilaments in the tips of hyphae may act as a load bearing structure. This reinforcing function would be important in turgor-driven growth. Heath (1995) suggested that the actin cytoskeleton may absorb some of the pressure generated by the turgid cytoplasm. This would assist in the maintenance of the shape

and health of the hyphal tips under pressure. It has also been hypothesised that the role of actin in cell elongation may be similar to that of the actin cytoskeleton in amoeboid cells (Harold et al 1995). In organisms employing amoeboid movement, polarized actin polymerization is thought to provide the driving force behind the protrusion and subsequent traction force required for cell extension and translocation of the cell (Pantaloni et al, 2001). This mechanism is not exactly analogous to the amoeboid-movement theory of hyphal extension (see Chapter 1 for details) but similar mechanisms might be employed.

No matter by which method tip-growing cells elongate, the surface area of the cell increases. This requires cell wall components to be transported to the apex of the cell where the expansion is occurring. The results of inhibitor studies have been used to establish a third role for the actin cytoskeleton in hyphal tip growth. This involves the actin network controlling the spatial and temporal positioning of secretory vesicles. With the addition of Cytochalasin (an inhibitor of F-actin extension) a swelling of the tip is observed (Heath, 1995; Grove and Sweigard, 1996, Torralba et al, 1998). Initially this was considered a function of the inability of actin to stabilize the force of the turgor pressure on the cell wall. Subsequently it was demonstrated that this facilitated a change from anisotropic to isotropic growth in some hyphal species (Torralba et al, 1998). These results support the theory that actin guides vesicles to the apical secretion site.

Actin plaques are present in hyphae. They are generally thought to be patches of undifferentiated actin attached to the cell membrane (Kaminskyj and Heath, 1996). In oomycetes the actin array is intimately associated with the plasma membrane. In true fungi, hyphae have been shown to contain peripheral plaques which are concentrated at the extreme tip or $\sim 5 \mu\text{M}$ behind the tip. The fibrils observed in these species are located centrally in the cells (Heath, 1990). The function of the plaques has not been established but there are thought to be species-specific differences between the roles they play. In oomycetes, plaques appear to be patches of actin associated with the plasma membrane displaying no obvious differentiation (Kaminskyj and Heath, 1996; Bachewich and Heath, 1997a). The role of the sub-apical fibrils has not been widely examined but they appear to be associated with the plaques in some species but not others. Their function remains undetermined.

None of these theories regarding the role of actin in tip growing cells are mutually exclusive (Heath, 1990). It is possible that under some conditions the actin cytoskeleton does provide mechanical support against the turgor pressure of the protoplast. It is equally as likely that it provides the driving mechanism behind pseudopodial movement, as hyphal growth has been shown to occur in the absence of hydrostatic pressure (Heath and Steinberg, 1999). There is little experimental data regarding vesicular transport and the filtering network roles of actin but these hypotheses provide plausible models for the cellular processes and organization observed in hyphal tip growth.

A variety of methods have been used to study the presence and distribution of actin in a range of fungal species (Heath, 1987, 1988; Schafer, 1998; Srinivasan et al, 1996). These techniques include biochemical and genetic analysis, light and electron microscopy, and inhibitor studies. Many of these methods have serious limitations and only by combining multiple techniques can definitive models of actin structure and function be developed. The techniques used to study the structural organization of the F-actin cytoskeleton in hyphal tips are currently limited to the introduction of fluorescent labels into the cells of interest. The most common of these techniques is the introduction of fluorochrome-labelled phalloidin or fluorescent antibodies. These types of techniques suffer from some major problems which include resolution, sensitivity and morphological variation caused both by the preparation and treatment of the samples. The resolution of the images obtained using the fluorescent labels is light-limited, meaning that some fine details of the actin network may be lost. Sensitivity can be impaired by background fluorescence. This may lead to structural features such as diffuse actin networks permeating the cytoplasm being obscured by the background interference. Artefactual results are a major concern as structural organisation is a key clue in determining the role the actin cytoskeleton plays in polarized cell extension. The fluorescent labels are typically introduced into the cells after they have been electroporated or fixed using a variety of buffers and/or detergents. Fixing or permeablizing cells will affect the labile arrays of actin in the cell (Kaminskyj et al, 1992). To some extent this can be circumvented by introducing the labels into actively growing cells (Jackson and Heath, 1990) and by improving the quality of fixation (Yu et al, 2004). An improved chemical fixation procedure has

recently been developed using methylgloxal in combination with formaldehyde (Yu et al, 2004). Methylgloxal has two functional aldehyde groups which react with amino acid residues forming stable cross links. Formaldehyde has only one functional aldehyde group. In combination, these aldehydes effectively preserve cells and allow visualisation of cellular structures such as the cytoskeleton. Due to the development of this method of fixation, the visualisation of previously unreported structures has been achieved.

The organisation of actin in hyphae displays intercellular and interspecific variations. This variability combined with difficulties in quantifying the results complicates the analysis of actin networks visualised within cells. Analyses that have been carried out so far have been based on easily observable differences between morphologically similar cells. In recent years it has become apparent that there are two distinct patterns of actin present in the hyphal tips of oomycetes (Jackson and Heath, 1990; Yu et al, 2004). This is characterised by an actin deplete zone (ADZ) in the extreme apex of healthy hyphal cells. The ADZ appears as a small area at the tip of the cell that does not fluoresce upon staining of the cell with flurochrome-labelled phalloidin. Besides the ADZ, the organization of the actin in the cells appears to be similar to those that do not contain the ADZ. The actin cap, sub-apical plaques and fibrils are apparently unaffected. The evidence for this in hyphal organisms is limited to two observations in oomycetes (Jackson and Heath, 1990; Yu et al, 2004). Experiments that have been carried out during the course of this thesis have helped confirm the presence of the ADZ in the hyphae of *Achlya bisexualis* and provided evidence regarding a functional role associated with it.

Materials and Methods

Materials and methods were as described in Chapter 2.

Results

Fixation of hyphae.

An initial experiment was carried out to determine which fixative most effectively preserved both the hyphae embedded in low melting point agarose (i.e. those growing invasively) and those that were growing on the surface of the agar (i.e. those growing non-invasively). The best fixative for both the invasive and non-invasive hyphae was found to be that containing (v/v) 0.5% methylgloxal, 4% paraformaldehyde, 50 mM PIPES pH 6.8, and 5 mM EGTA. The quality of fixation was assessed qualitatively by examining hyphal shape and the appearance of the cytoplasm subsequent to the fixation procedure. Good fixation was indicated by a tapered tip and a well preserved cytoplasm that was devoid of major discontinuities as is observed in healthy, growing hyphae. Figure 3.1 shows representative examples of unfixed hyphae. Figure 3.2 shows representative examples of well fixed hyphae. Figure 3.3 shows representative examples of badly fixed hypha.

Staining of invasive and non-invasive hyphae.

After fixation and subsequent staining with Alexa Phalloidin, an actin deplete zone was observed in the invasive hyphae of some samples. This appeared as an area exhibiting little or no fluorescence at the extreme apex of the cell. Figure 3.4 shows hyphae containing the actin deplete zones. Figure 3.5 shows hyphae where the actin deplete zones were not present. In these hyphae, the actin network forms a continual fibrillar cap at the cell tip.

Initially the hyphae were prepared using the overlay technique which is detailed in Chapter 2. Of the seven experiments carried out in 1% LMP agarose, three had significantly higher numbers of actin deplete zones in the invasive sample compared to the non-invasive samples ($P > 0.05$). An average 54% of the invasive and 9% of the non-invasive hyphae in these samples had ADZs. Of the seven experiments carried out in 4% agarose, two had significantly higher numbers of actin deplete zones in the invasive sample compared to the non-invasive sample ($P < 0.05$). An average

49.5% of the invasive and 5% of non-invasive hyphae in these samples had ADZs. These results are summarised in Table 3.1. Figure 3.6 contains images of the deplete zones obtained using this technique. The reason for the appearance of the ADZ in the invasive hyphae in some experiments but not others has not been established. The method of preparation of the samples was altered slightly in an attempt to ascertain the underlying cause of these variable results. This included varying temperature, the length of time the LMP agarose was boiled for prior to application and the incubation time of the cultures. None of these factors were found to increase the prevalence or predictability of the occurrence of the actin deplete zone.

A different preparation technique was therefore trialled (as described in the fixation and staining sections of Chapter 2) resulting in actin deplete zones being consistently observed in invasive hyphae but not non-invasive. Both experiments yielded significantly higher percentages of actin deplete zones in the invasive compared to the non-invasive samples ($P < 0.001$). An average of 63% of the invasive and 12% of the non-invasive hyphae in these samples had ADZs. See Table 3.2 for details and Figure 3.7 for images of the deplete zones obtained using this technique. The hyphae were imaged using DIC, epifluorescent and confocal methodologies. Both epifluorescent and confocal microscopy were used to ensure that the focal plane of the epifluorescent microscope was not the reason for the observation of the ADZ. Due to time constraints this was only carried out twice but the technique has subsequently (in 5 out of 5 experiments) yielded significantly higher proportions of invasive hyphae containing actin deplete zones relative to non-invasive hyphae (Chitcholtan, 2004).

In order to establish that the technique consistently enabling the visualisation of the actin deplete zones in the invasive hyphae was not just a coincidence of timing, both the techniques were carried out concurrently. This yielded results suggesting the technique in which hyphae are embedded in 2% low melting point agarose is superior for the imaging of the actin deplete zone that the overlay technique. In the sample prepared using the overlay technique, 1% of the invasive hyphae had ADZs whereas 88% of the invasive hyphae prepared using the embedding technique had the deplete zone. This difference is statistically significant ($P < 0.001$). See Table 3.3 for details.

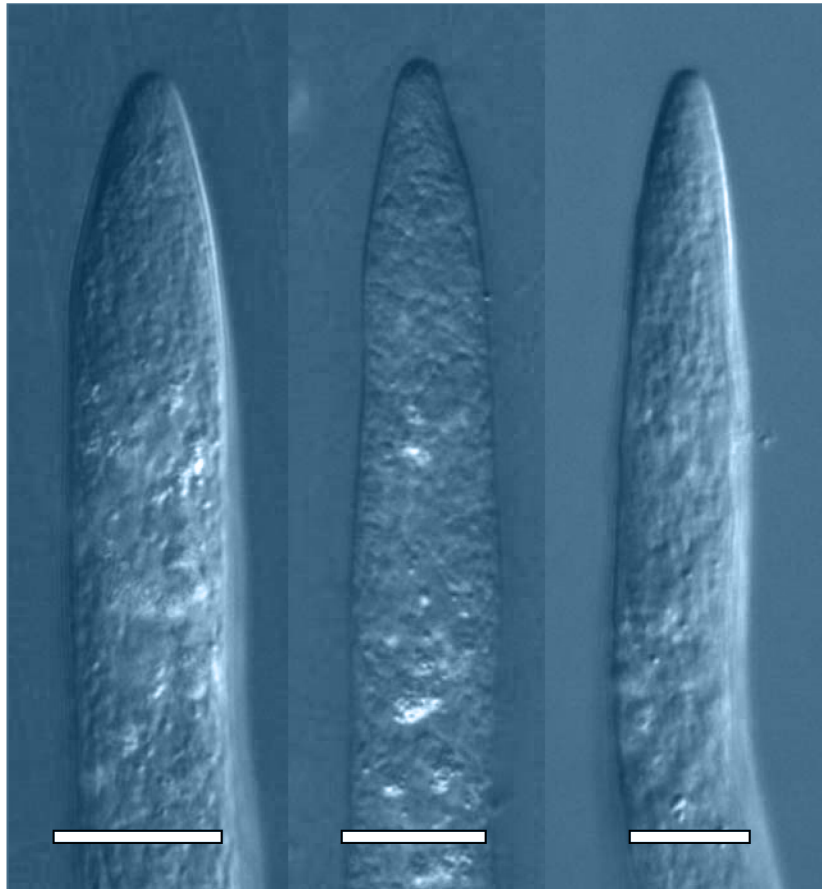


Figure 3.1 - Unfixed hyphae of *Achlya bisexualis* with tapered tips and cytoplasm devoid of any major discontinuities. These images were acquired using DIC optics and captured using a Coolsnap camera. See Chapter 2 for details. Bar = 10 μ M.

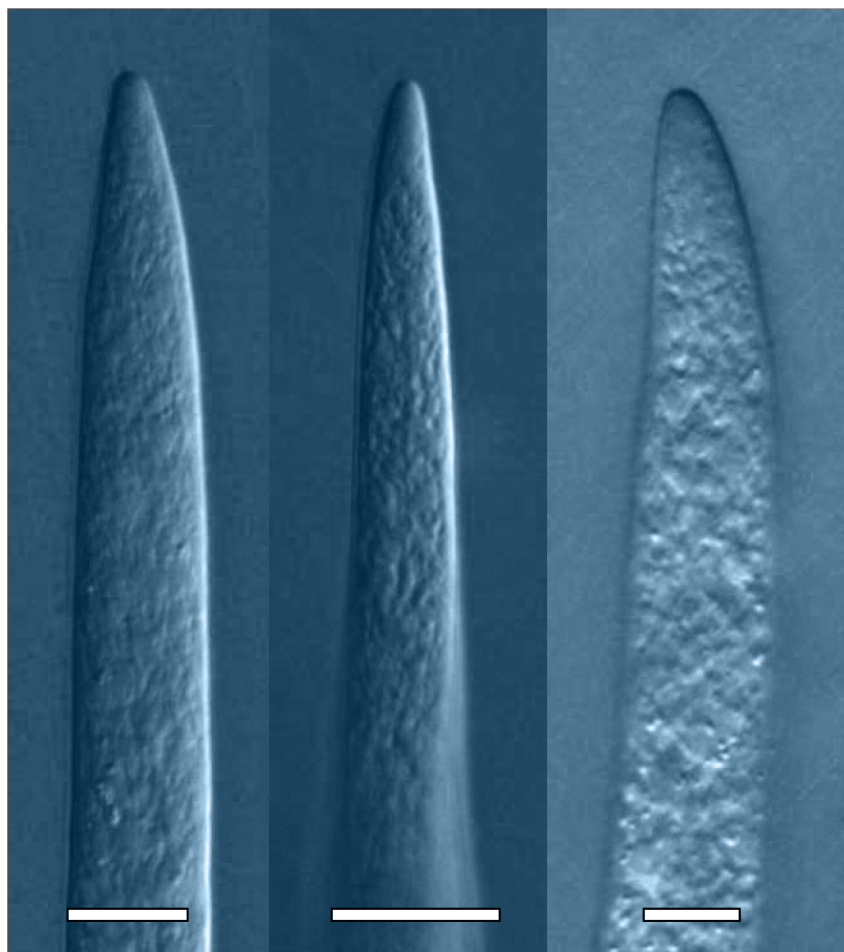


Figure 3.2 – Hyphae of *Achlya bisexualis* fixed with (v/v) 0.5% methylgloxal, 4% paraformaldehyde, 50 mM PIPES pH 6.8, and 5 mM EGTA. The tapered tips and cytoplasm devoid of any major discontinuities were considered indicative of good fixation. These images were acquired using DIC optics and captured using a Coolsnap camera. See Chapter 2 for details. Bar = 10 μ M.

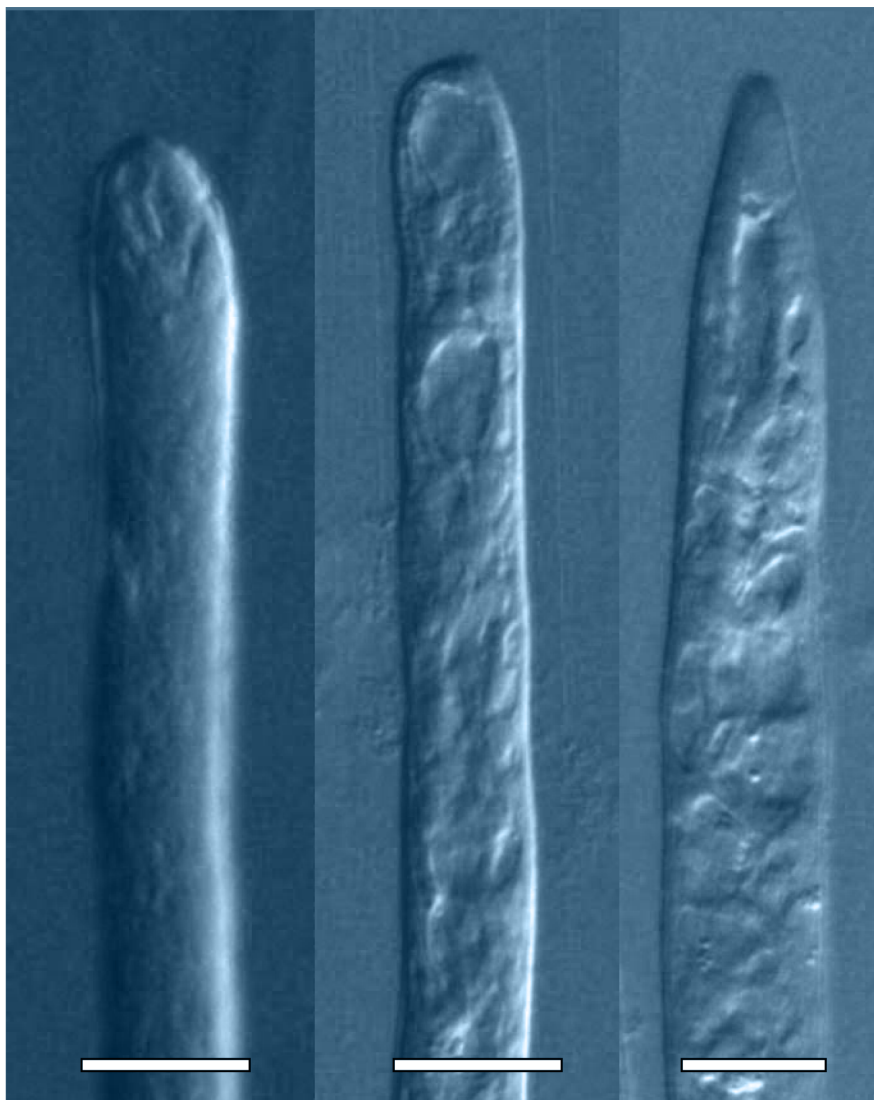


Figure 3.3 – Hyphae of *Achlya bisexualis* fixed with (v/v) 0.5% methylgloxal, 4% paraformaldehyde, 100 mM PIPES pH 6.8, and 0 mM EGTA. The rounded tips and the major discontinuities in the cytoplasm devoid were considered indicative of bad fixation. These images were acquired using DIC optics and captured using a Coolsnap camera. See Chapter 2 for details. Bar = 10 μ M.

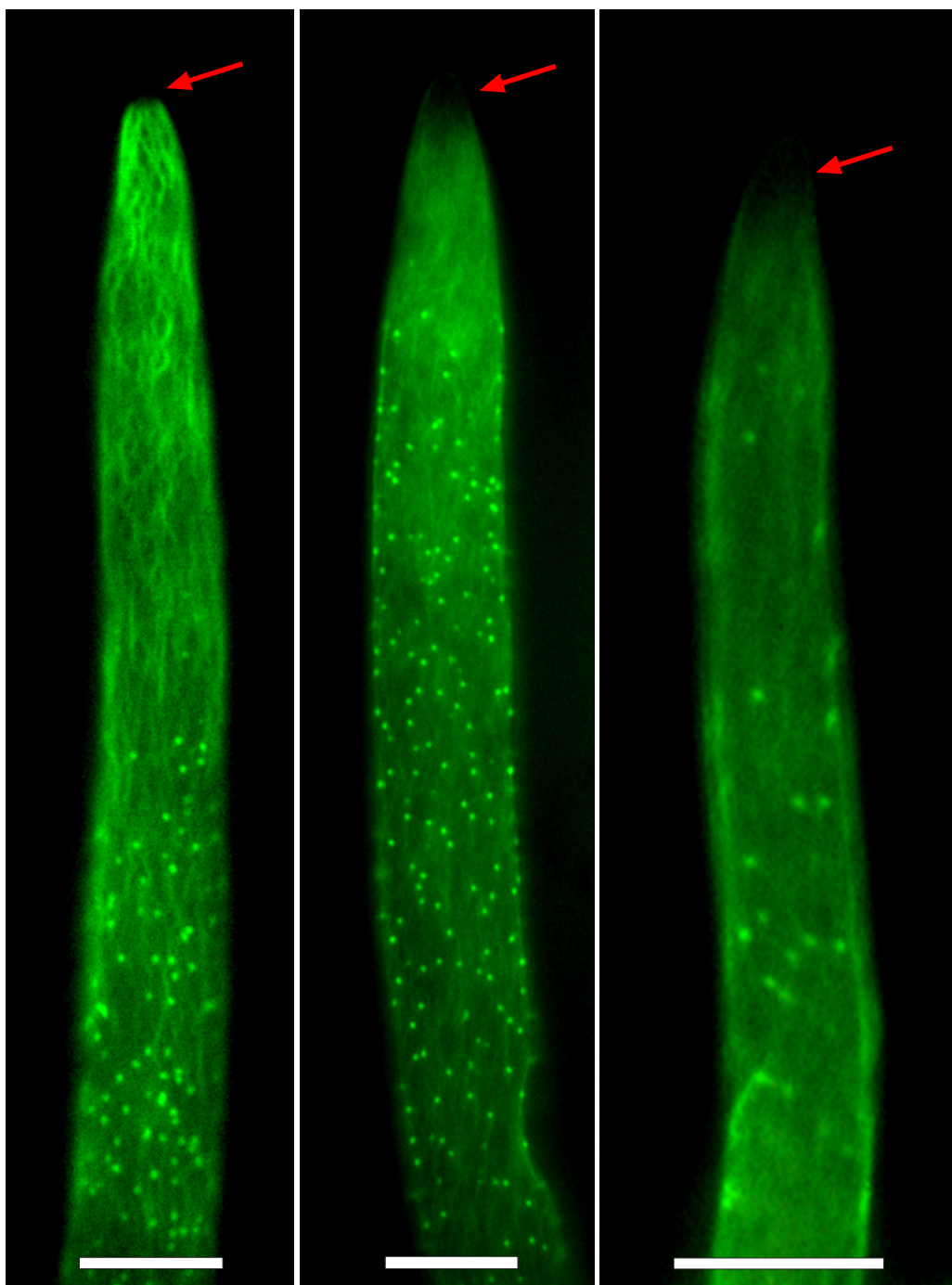


Figure 3.4 - After fixation and subsequent staining of *Achlya bisexualis*, actin deplete zones were observed in the invasive hyphae. These appeared as areas exhibiting little or no fluorescence at the extreme apex of the cell as indicated by the red arrows. These epifluorescent images were obtained using techniques detailed in Chapter 2. Bar = 10 μm .

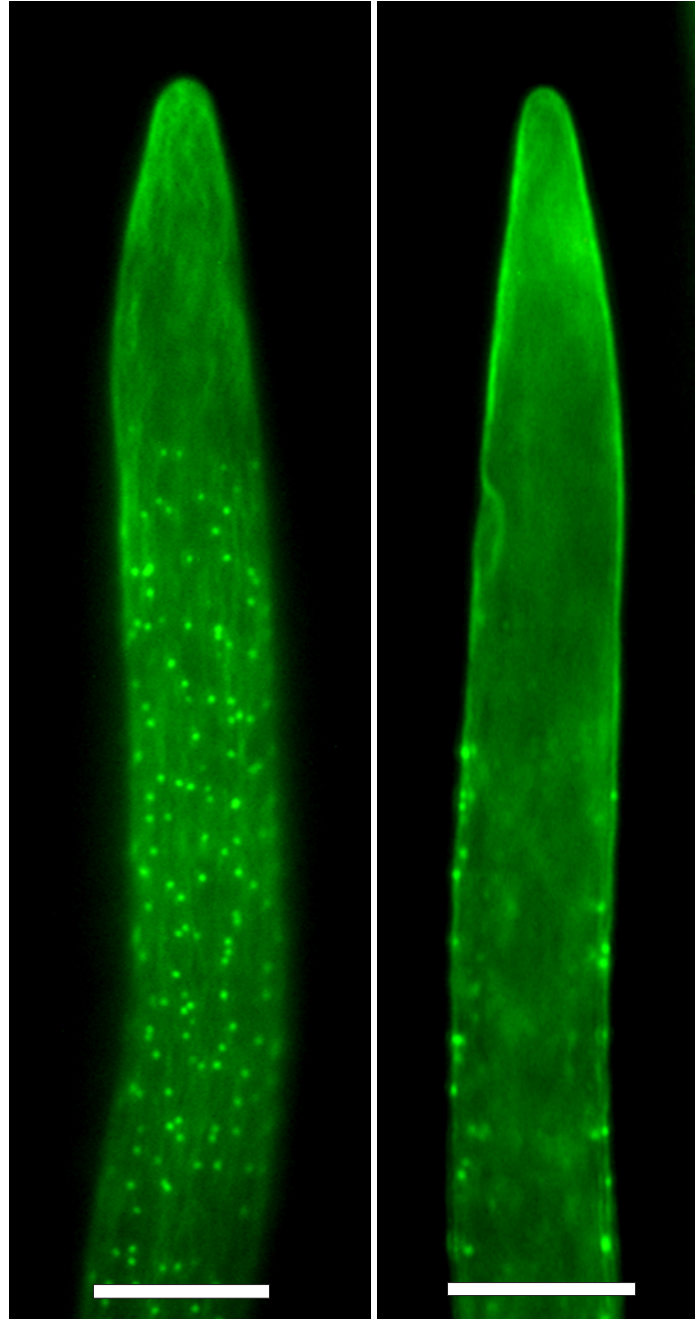


Figure 3.5 - After fixation and subsequent staining of *Achlya bisexualis*, complete actin caps were observed in non-invasive and some invasive hyphae. These are characterised by a network of fibrillar actin that extends to the hyphal tip and a subapical region of interconnected plaques and fibrils. The distribution of actin in these images appears dissimilar due to the position of the focal plane. These epifluorescent images were obtained using techniques detailed in Chapter 2. Bar = 10 μm .

% agar	% with ADZ in the non-invasive sample	% with ADZ in the invasive
1	4.54	75.0
1	0.0	28.6
1	22.2	60.0
1	0.0	6.9
1	1.18	1.1
1	0.0	0.6
1	0.0	0.6
4	0.0	20.0
4	9.68	79.0
4	0.0	0.0
4	0.0	0.0
4	0.0	0.0
4	1.49	2.6
4	0.0	1.2

Table 3.1 – Overlay technique results. Seven experiments were carried out in 1% LMP agarose of which three yielded significantly higher numbers of actin deplete zones in the invasive sample compared to the non-invasive sample ($P < 0.001$). Seven experiments were carried out in 4% agarose of which two contained significantly higher numbers of actin deplete zones in the invasive sample compared to the non-invasive sample ($P < 0.001$).

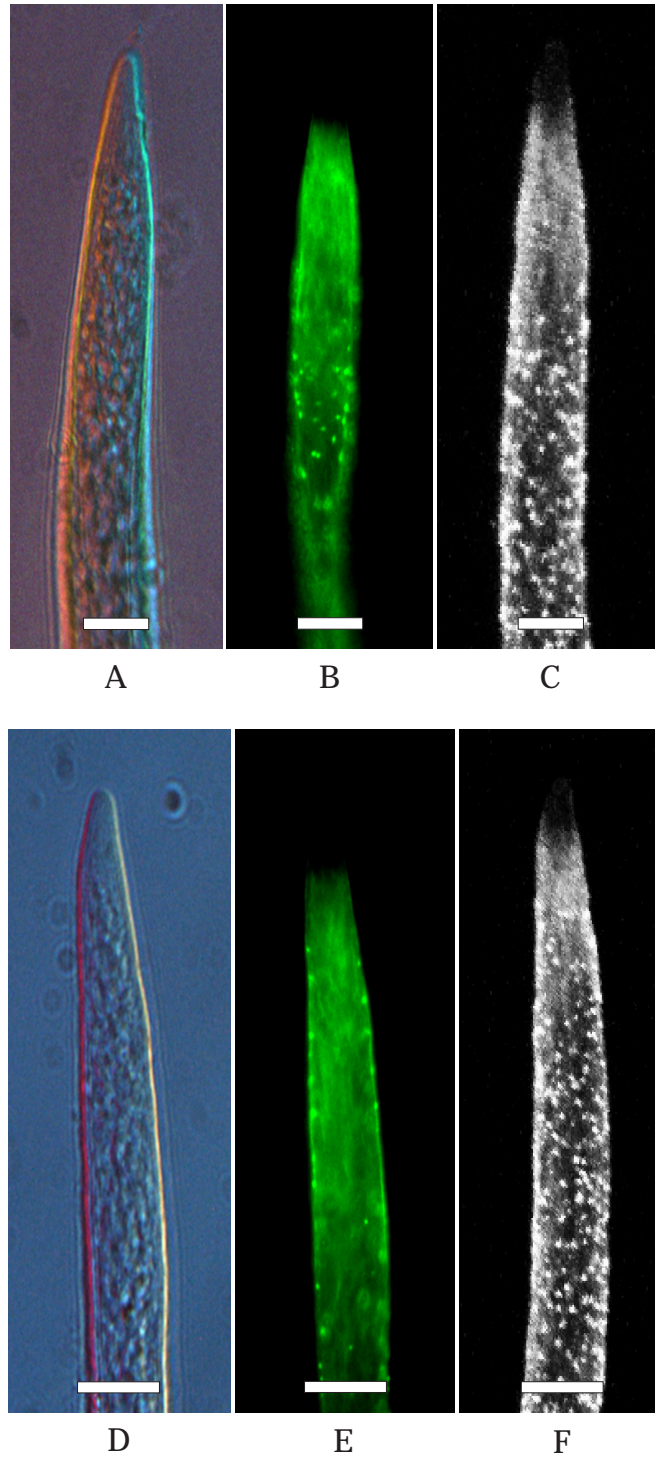


Figure 3.6 - After fixation and subsequent staining of *Achlya bisexualis*, actin deplete zones were observed in the invasive hyphae. These images were obtained from a sample prepared using the overlay technique. Images A and D are DIC images. Images B and E are epifluorescent images. Images C and F are confocal images. See Chapter 2 for details regarding the techniques. Bar = 10 µm

% agar	% with ADZ in the non-invasive sample	% with ADZ in the invasive
2	0.00	67.0
2	23.71	59.0

Table 3.2 – Embedding technique results. Two experiments were carried out using 2% LMP agarose of which both had significantly higher numbers of actin deplete zones in the invasive sample compared to the non-invasive sample ($P < 0.001$). The reason for the disparity between the non-invasive samples is not known.

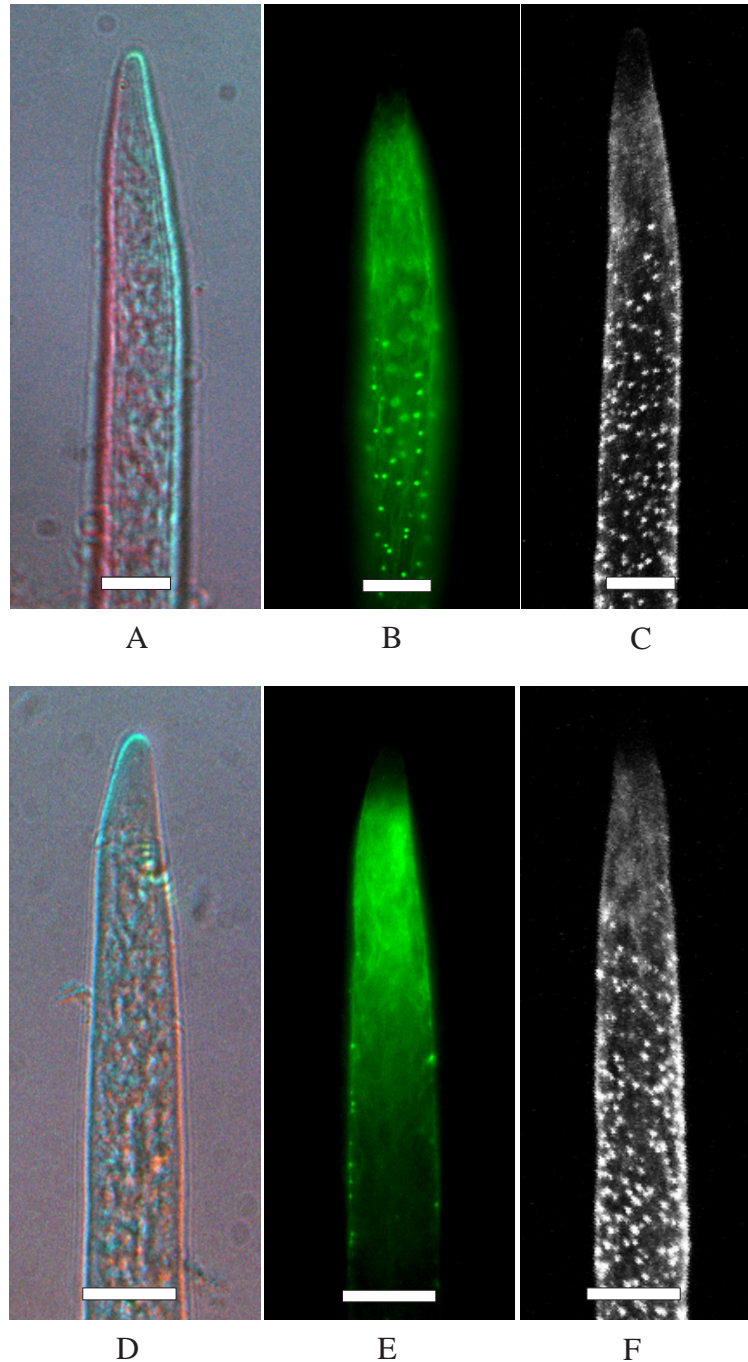


Figure 3.7 - After fixation and subsequent staining of *Achlya bisexualis*, actin deplete zones were observed in the invasive hyphae. These images were obtained from a sample prepared by embedding the hyphae in 2% LMP agarose. Images A and D are DIC images. Images B and E are epifluorescent images. Images C and F are confocal images. See Chapter 2 for details regarding the techniques. Bar = 10 μ m.

% agar	% with ADZ in the non-invasive sample	% with ADZ in the invasive
2	0.6	88.2
1	0.0	0.9

Table 3.3 – Concurrent embedding and overlay technique results. The experiment carried out in 2% agarose corresponds to the embedding technique and yielded significantly higher numbers of deplete zones in the invasive compared to the non-invasive sample ($P < 0.0001$). The experiment carried out in 1% agarose corresponds with the overlay technique and while the invasive sample contained a higher percentage of deplete zones, this difference was not statistically significant ($P < 0.01$).

Discussion

The presence of an actin deplete zone in the tips of oomycete hyphae is somewhat contradictory to most of the research published regarding actin organisation in these polarised cells. Historically it has been accepted that the actin network is a continuous cap of filamentous actin extending to the very apices of the cells with subapical arrays of plaques and interconnecting fibrils. These structures are thought to carry out several essential roles including influencing overall hyphal morphogenesis, cellular polarity, structural support, and facilitating the transport of exocytotic vesicles to the hyphal apex.

In recent years there have been papers published in which actin deplete zones have been observed in a number of types of tip growing cells (Jackson and Heath, 1990; Yu et al, 2004) though their occurrence is cited as being unpredictable and few suggestions had been made regarding the underlying cause. Pollen tubes are tip growing cells in which actin deplete zones have been most often observed. It has been suggested that this region is an area in which the polymerisation of microfilaments occurs (Vidali and Hepler, 1990) though this hypothesis remains unconfirmed. Hyphae of oomycetes have been found to contain actin deplete zones (Jackson and Heath, 1990 and Yu et al, 2004). The lack of fluorescence in the apical zone of these cells suggests that there are no microfilaments in these areas as phalloidin based probes only bind to and fluoresce polymerised F-actin.

During the course of this thesis significantly higher numbers of actin deplete zones have been observed in invasive compared to non-invasive hyphae. These data are exciting with regard to further elucidation of the role of the cytoskeleton in hyphal tip growth. The presence of this actin deplete zone in the invasive hyphae suggests that a slightly different mode of growth is employed in these cells and that the cytoskeleton does not reinforce the cell apex to the same extent in non-invasive hyphae. In the paper by Yu et al (2004) it was suggested that the actin deplete zone may be associated with an area that yields more readily to turgor pressure or protrusive force. It was also hypothesised that this structure may enable the exclusion of larger organelles at the tip and could represent a major site of exocytosis of wall materials as

observed in root hairs (Ketelaar, 2003). The exact role of the actin deplete zone in these cells remains unclear but with further investigation an elucidation of mechanisms of hyphal extension may be achieved.

The quality of fixation of the hyphae prior to staining is thought to have a major influence on the structures within the actin cytoskeleton. It has only been with the development of improved staining techniques that the actin deplete zone has been visualised (Yu et al, 2004; Jackson and Heath, 1990). Major assumptions are still made with regard to these techniques in that it is assumed that the observed structures are replicas of the organisation of actin in living hyphae. It is possible however that the process of chemical fixation induces the cytoskeleton to rearrange, causing a difference in the organisation of actin in living cells compared to that of fixed cells. Imaging of the cytoskeleton in live cells would be ideal however the introduction of labels and probes into live fungal cells has proved difficult.

The actin deplete zone appears to be an unstable and/or extremely dynamic structure as on many occasions it was not observed. Though attempts were made to elucidate the exact cause for the variability in occurrence, no conclusion could be drawn from the experiments carried out (Jackson and Heath, 1993). The fact that the initial overlay method produced variable results suggested that either the actin deplete zone was an artefact of the technique or it was a fragile structure which is altered rapidly in response to environmental stimuli. These hypotheses are somewhat contested in that when the deplete zone was observed it occurred at significantly higher frequencies in the hyphae growing invasively. The alternative method of fixing and staining produced far more consistent results always yielding higher frequencies of actin deplete zones in the invasive samples. The presence of deplete zones in the non-invasive hyphae could be attributed to variable staining although this seems unlikely as the vast majority of the hyphae in the samples were well stained. As it was occasionally difficult to distinguish the invasive from the non-invasive hyphae, it is possible that the non-invasive hyphae containing deplete zones could have actually been partially or fully embedded in the agarose and therefore employing invasive growth mechanisms. A similar situation could be used to explain the lack of deplete zones in the invasive hyphae.

To strengthen the case for the presence and role of actin deplete zones in hyphal organisms, it needs to be identified in other hyphal species including true fungi. Due to time constraints, experiments investigating the cytoskeleton in *Neurospora crassa* and *Mortierella wolfii*, (species that have been investigated in Chapter 5 of this thesis) could not be carried out. The presence of the actin deplete zone in the hyphal apices of the oomycete *Achlya bisexualis* presents some very interesting and exciting avenues for further research regarding the mechanism of cytoskeletal assisted polarised extension of hyphae.

CHAPTER 4

Cell pressure

Like other eukaryotic organisms, fungal cells contain an internal concentration of solutes higher than that of their surroundings (Adebayo et al, 1971). This results in the flow of water into the cell such that an internal hydrostatic pressure or turgor is generated (Eamus and Jennings, 1986a). The action of turgor pressure on the cell wall has long been considered essential in hyphal extension (Harold et al, 1996; Bartnicki-Garcia, 2000; Wessels, 1986; Heath, 1995).

These ideas originate from those of Lockhart (1965) which applied to plant cell growth. Lockhart believed that in plants, as is now commonly accepted, water uptake is driven by differences in water potential between the cell and surroundings, and that the cell wall yields to this turgor pressure (Ray and Green, 1972). In this sense turgor appears to drive expansion. If this were the case, the rate of increase of the volume of the cell should be proportional to the rate of water influx. This has been described mathematically using several formulae (Lockhart 1965, Ortega 1985 and 1988, Jennings, 1991). These equations predict that changes in growth rate are associated with changes in extensibility, turgor and yield threshold of the cell wall. More recently however, increasing amounts of data indicate that in hyphae the relationship between the turgor pressure and growth is not as clear as these models would suggest. Furthermore an additional problem associated with numerical descriptions of hyphal growth is that, while in some cases they accurately predict growth rate, they do not explain or account for all growth processes (for example, it is unclear how to define the action of wall softening agents (endoglucanases) or the development of cross linking between cell wall polymers). An additional problem regarding the use of the Lockhart equations is that they were initially formulated for plant cells and may therefore be inapplicable to fungal cells.

The question then arises as to what role turgor plays in fungal/oomycete biology and what drives growth (Money, 1994, 1997, 1999b, 2001; Money and Harold, 1992). With regard to the latter question it is worth considering the ideas of Reinhardt who in 1892 hypothesised that turgor was necessary to press the membrane against the inside of the cell wall but that this did not necessarily drive cell extension. The alternate mechanism proposed by Reinhardt's contemporaries was termed the amoeboid movement theory (Heath, 1995; Heath and Steinberg, 1999, Pickett-Heaps and Klein, 1998). This model likens hyphal extension to the movement of tube dwelling amoebae. This involves the cytoplasm at the apex of the cell being protruded like a pseudopodium. The protrusion then adheres to the substrate by means of transmembrane integrin-like proteins (Hynes, 1992; Kaminskyj and Heath, 1995). A traction force must then be exerted by the means of the cytoskeleton. This would move the bulk of the cytoplasm towards the pseudopodium. Unlike true amoeboid movement hyphal extension does not require any translocation of the sub-apical material but the supplementation of the pre-existing cytoplasm (Heath and Steinberg, 1999). The wall components are then exocytosed by the means of vesicles and assembled on the surface of the cell. This type of growth would, in theory be possible without internal hydrostatic pressure. This raises the question of whether tip growth is possible in the absence of turgor.

The most pertinent experiments with regard to the above question are those carried out on the water molds, *Achlya* and *Saprolegnia* (Kaminskyj et al, 1992, Money and Harold, 1993). Neither of these organisms appear to have the ability to turgor regulate which means that their cellular turgor pressure is essentially set by the water potential of the solution surrounding the cells. Experiments have shown that the point at which the osmotic potential of the growth medium reaches -0.80 MPa, turgor in *Achlya* cells is unable to be measured using a pressure probe. These devices are not able to accurately measure pressures lower than 0.02 MPa which means that the pressure within those cells must be at or below this value. Significantly it was observed that these hyphae continued to extend in a polarised manner, albeit at a much reduced rate. The wall strength in these cells was reduced by approximately 90% upon the reduction of turgor. *Saprolegnia* also exhibits polarised tip growth in the absence of measurable turgor as measured with a cell pressure probe. In this case when the osmotic potential of the media reaches -0.6 MPa the pressure of the cell

decreases below 0.02 MPa. Under these conditions tip extension of *Saprolegnia* hyphae continues and the rate actually increases between osmotic potentials of -0.05 and -1.0 MPa with an apparently linear relationship between turgor and wall strength. This is consistent with the hypothesis that the yielding capabilities of the wall (and possibly the cytoskeleton - see Chapter 2), not turgor pressure per se are the important determinants of growth. This in turn raises the question as to why cells generate turgor as it is energetically expensive to maintain. It is possible that turgor is maintained for other cellular processes such as growth through solid substrates, the establishment and function of reproductive structures and translocation of solutes through the mycelium.

Often solutes or water are required to be transported across the membrane against the concentration gradient. This uses energy in the form of ATP suggesting the need for a distinct evolutionary advantage in the maintenance of turgor (Money, 2001). Turgor is understood to maintain form and stiffness of some fungal cells and other multicellular structures associated with successful growth and reproduction (Money and Webster, 1988). The importance of turgor can be illustrated in the interplay of internal pressure and cell wall strength that underlies the formation of fruiting bodies in some species of fungi (Hoch and Mitchell, 1973). In this sense turgor almost performs a skeletal function, the pressurised cell contents being referred to as the hydroskeleton (Vogel 1988.) The loss of turgor eliminates the hydroskeleton and spore release is often curtailed as the fruit body shrivels. However it should be noted that the fruiting bodies of some species of fungi can survive repeated desiccation and rehydration without losing their ability to produce and release spores and in some cases turgor appears to play only a minor role in the maintenance of cell shape (Holloway and Heath, 1977). In these cases it is thought that the cytoskeleton has the major role in supporting the cell wall (see Chapter 2).

It has been demonstrated that turgor is an important factor in the deformation and penetration of solid substrates in some species of filamentous fungi. The rice blast pathogen *Magnaporthe grisea* produces highly pressurised appressoria which puncture plant cell walls. These structures have turgor pressures of 8.0 MPa or more (Howard et al, 1991; Howard and Valent, 1996). It is thought that this provides the means for the fungus to invade the host. Turgor, along with the excretion of

hydrolytic enzymes (proteases), is also considered important in the mycotic infections of animal tissues. However knowledge of the biophysical aspects of penetration and growth through substrates remains limited. The exact role of turgor in this type of growth remains unconfirmed. The force applied by the hyphal tips is not only dependent on the intracellular turgor pressure but also the wall plasticity. No matter how high the turgor pressure gets, no substrate penetration can be achieved without yielding of the cell wall. The more the wall yields, the more force can be applied to the substrate. Therefore turgor, wall strength and the ability to penetrate may all be linked but until more research is carried out this speculation will remain unsubstantiated.

Experiments detailed in this chapter address the role of turgor in growth by measuring turgor in invasive and non-invasive hyphae in order to establish whether there are differences between the two modes of growth and how these might relate to the mechanisms of cell extension.

Materials and methods

Materials and methods were as described in Chapter 2.

Results

To determine whether turgor pressure differs between invasive and non-invasive hyphae, measurements were made directly using a cell pressure probe and indirectly using incipient plasmolysis. The pressure probe was also used to assess whether *A. bisexualis* is able to turgor regulate and for the measurement of wall strength.

Pressure probe turgor measurements

The measurement of invasive hyphal turgor necessitated movement of the glass pipette through agarose. This reduced the manual control over the movement of the pipette when approaching the hypha but meant that the insertion of the pipette into the invasive hypha was much easier than the non-invasive hyphae due to greater purchase on the cell. Upon initial immersion into solution, the surface tension forced the aqueous solution into the micropipette. Using the pressure probe apparatus, pressure was applied to the meniscus formed at the oil-aqueous solution interface, moving it to the tip of the micropipette. The pressure required to do this was used as the “zero” or “offset” pressure and was subtracted from the final pressure value in order to obtain the turgor pressure of the cell. Upon impalement of the hyphae with the micropipette, the oil-cytoplasm interface moved away from the micropipette tip. Positive pressure was then applied to the oil in order to bring the meniscus to the cell wall as shown in Figure 4.1. To ensure that the tip of the micropipette was not plugged, the meniscus was drawn back from the micropipette tip through the application of negative pressure using the pressure probe. The meniscus was then returned to the cell wall. This was carried out several times during the course of an experiment. For an experiment to be considered successful and used as results, this was carried out at least twice and in the majority of cases 4-5 times per cell. The pressures required to return the meniscus to the cell wall were used to estimate the hyphal turgor pressure. During some measurements, cytoplasmic movement could be seen occurring within the hyphae punctured with the micropipette. Tip growth was also observed during the course of some experiments. On some occasions a cytoplasmic plug would form around the point of insertion of the micropipette. An example of a cytoplasmic plug is shown in Figure 4.2. In these cases plugging of the tip of the micropipette was common and the experiments were aborted. Upon removal of the micropipette from the cell, leakage

of cytoplasm from the site of impalement and/or oil from the micropipette was considered indicative of a successful pressure recording unimpeded by the wounding response. The turgor pressures of both invasive and non-invasive hyphae were measured. The mean pressures of the invasive hyphae were 6.41 ± 0.68 bars (range; 4.33 – 7.41 bars). The pressures of the non-invasive hyphae were 5.66 ± 1.62 bars (range; 2.6 – 8.1 bars). The respective invasive and non-invasive turgor pressures were not significantly different ($P < 0.05$). Representative pressure traces obtained from these experiments are shown in Figures 4.3 and 4.4.

Turgor regulation

Using osmotic treatments in combination with pressure probe measurements it was found that *A. bisexualis* does not turgor regulate. Upon hyperosmotic shock, turgor of the hyphal cells declined to near zero and did not usually recover though some partial transient recovery was observed in some continuous experiments as shown in Figure 4.5. What these results suggest is that the turgor pressure of the hyphal cell is determined by the osmolarity of the surrounding media.

Incipient plasmolysis

To determine the internal osmotic potential of the hyphae, the plasmolysis data were plotted using SigmaPlot 2000. These curves were fitted using the method detailed in Chapter 2. The sigmoidal curves obtained are shown in Figures 4.6 and 4.7. The hyphal turgor pressures obtained were 6.47 bars for the invasive hyphae and 6.24 bars for the non-invasive hyphae. These values are close to the measurements made with the cell pressure probe displaying no significant difference ($P < 0.05$), indicating that there was very little experimental error or variation between the direct and indirect techniques and that turgor does not differ significantly between invasive and non-invasive hyphae.

Burst pressures

To indirectly assess cell wall strength, the pressure probe was used to determine the burst pressures of invasive and non-invasive hyphae. The turgor pressures of hyphae

were obtained with the pressure probe using the method described above. The pressure was then increased such that oil moved into the hypha. The oil continued to be injected into the cell until the hyphae burst, at which point the pressure was recorded. The rupture point always occurred at the apex of the cell. The difference between the initial pressure measurement and that at which the cell burst was calculated. The pressures at which tip bursting occurred in the invasive and non-invasive hyphae were not significantly different ($P < 0.05$). The differences between the initial pressures and that at which bursting occurred were statistically significantly different ($P < 0.05$). The increase in pressure required to cause the tips to burst in the invasive hyphae was 2.41 ± 0.76 bars (range; 1.81 – 3.7 bars) and 3.75 ± 0.79 bars (range; 3.07 – 4.98 bars) for the non-invasive. These results are summarised in Table 4.1.

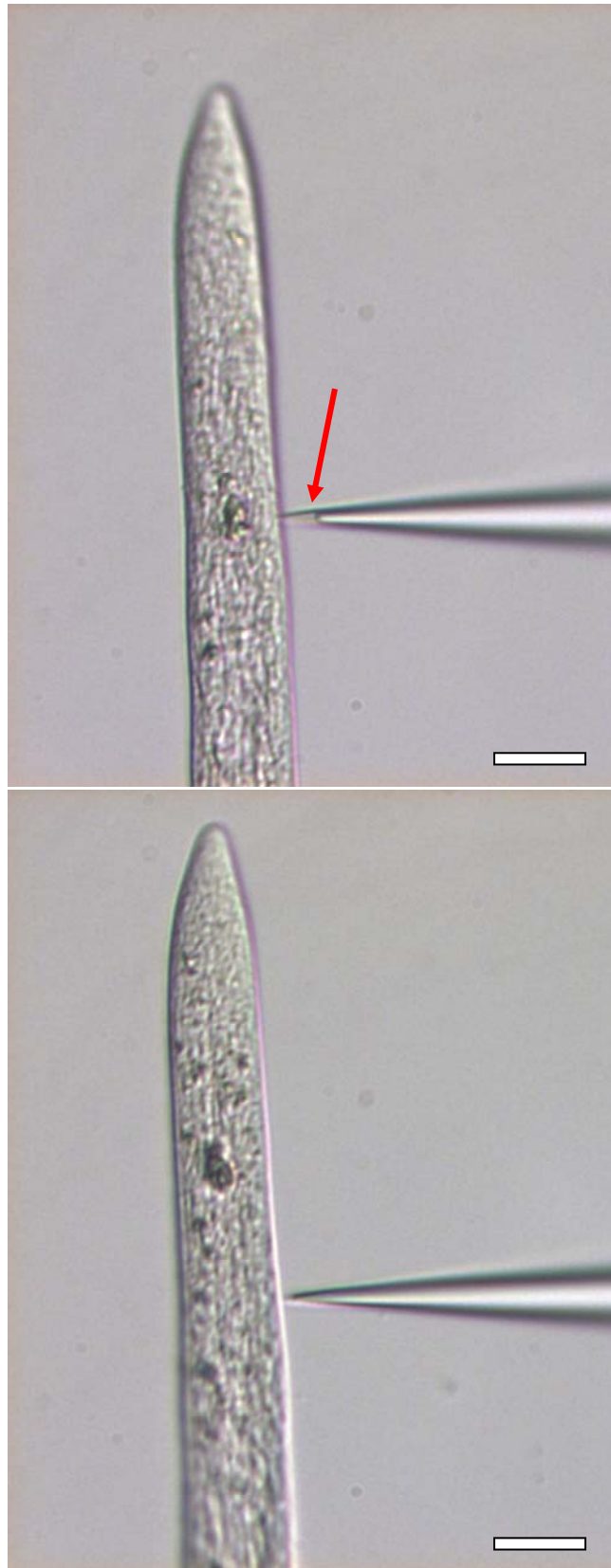


Figure 4.1 - Pressure probe measurements of *Achlya bisexualis*. The oil-cytoplasm meniscus (red arrow) has been brought to the cell wall through the application of positive pressure. Bar = 10 μm

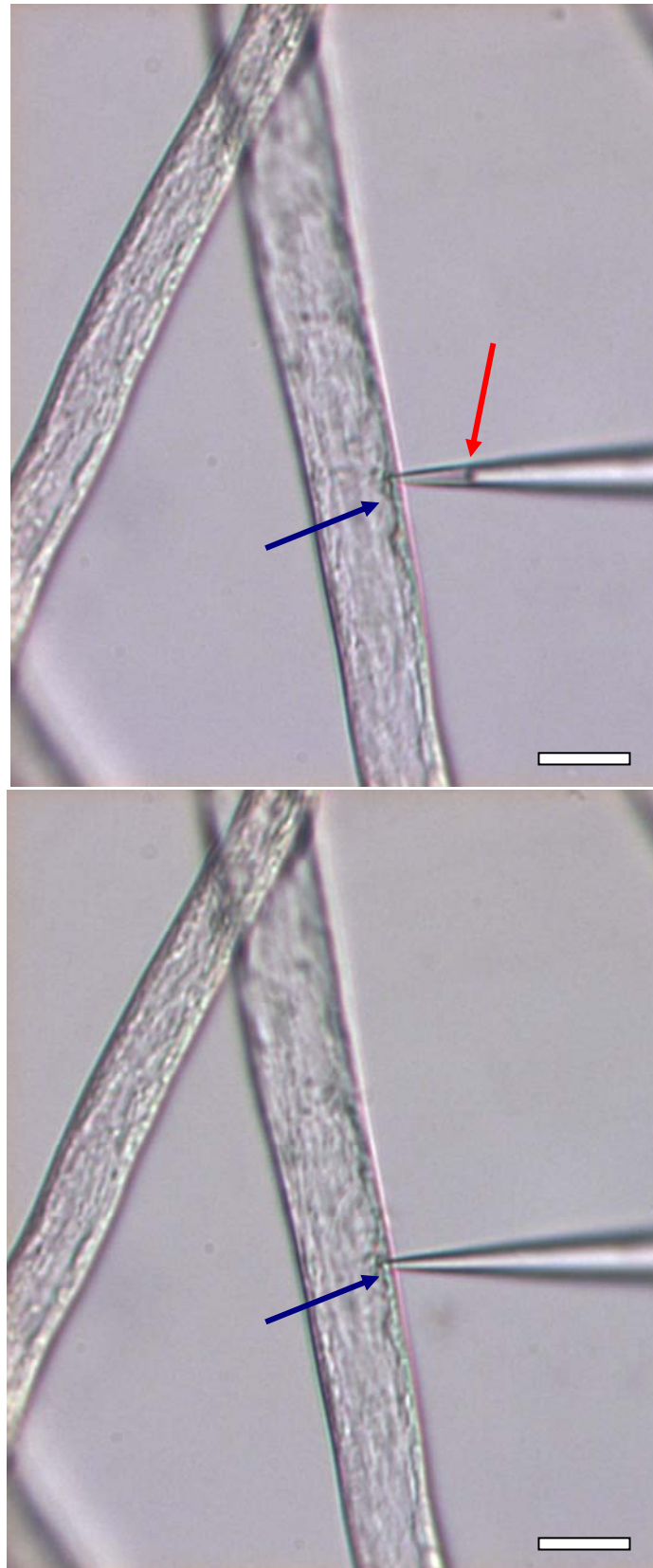


Figure 4.2 - Pressure probe measurements of *Achlya bisexualis*. The oil-cytoplasm meniscus (red arrow) has been brought to the cell wall through the application of positive pressure. The initial signs of the formation of a cytoplasmic plug are indicated by the blue arrows. Bar = 10 µm.

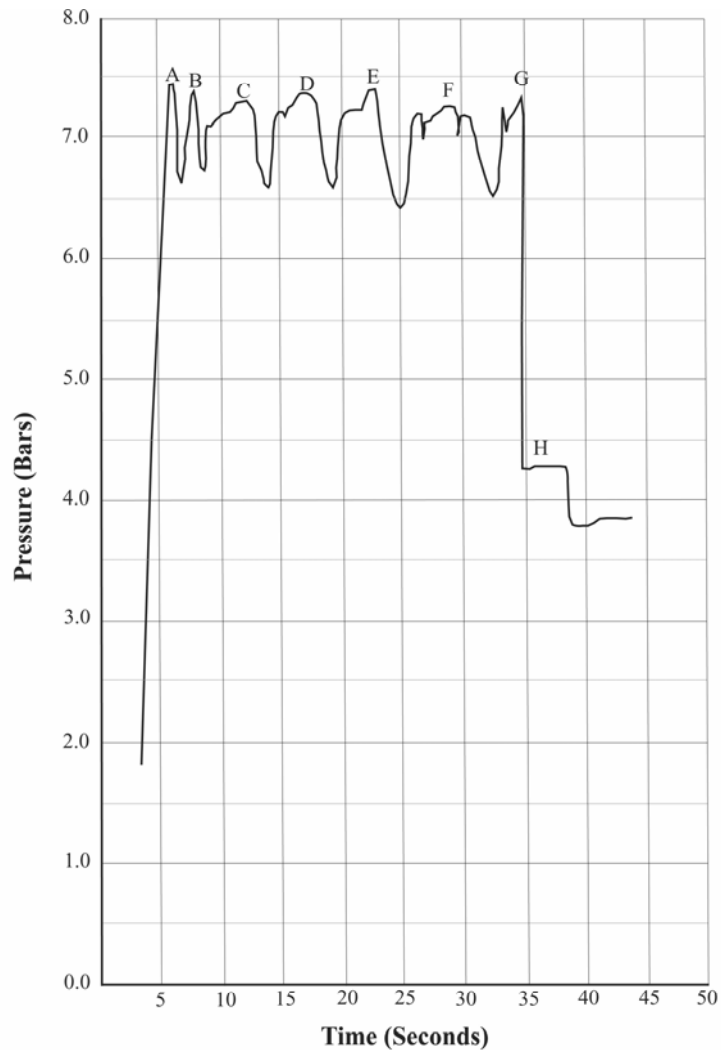


Figure 4.3 – Pressure probe recording of an invasive *Achlya bisexualis* hypha. The points marked A, B, C, D, E, F, and G are indicative of pressures at which the meniscus was at the cell wall and correspond to 7.46, 7.45, 7.41, 7.44, 7.44, 7.38, and 7.44 bars respectively. Subtracting the offset (1.8 bars) gives corresponding cell turgor pressures of 5.66, 5.65, 5.61, 5.64, 5.64, 5.58, and 5.64 bars. H indicates the point at which the micropipette was removed from the hypha causing the expulsion of cytoplasm from the cell and oil from the micropipette.

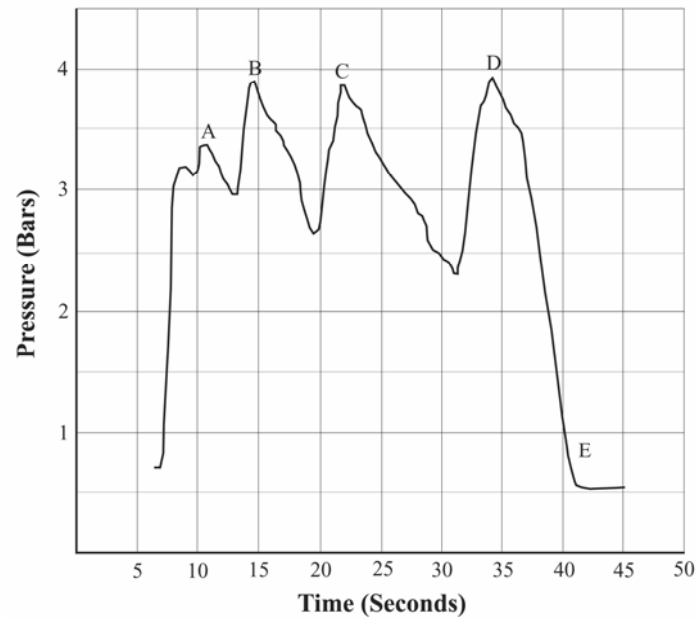


Figure 4.4 – Pressure probe recording from a non-invasive *Achlya bisexualis* hypha. The points marked A, B, C, and D are indicative of pressures at which the meniscus was at the cell wall and correspond to 3.3, 3.86, 3.84, and 3.9 bars respectively. Subtracting the offset (0.7 bars) gives corresponding cell turgor pressures of 2.6, 3.16, 3.14, and 3.2 bars. E indicates the point at which the micropipette was removed from the hypha causing the expulsion of cytoplasm from the cell and oil from the micropipette. Note that this pressure recording is from the lower end of the range.

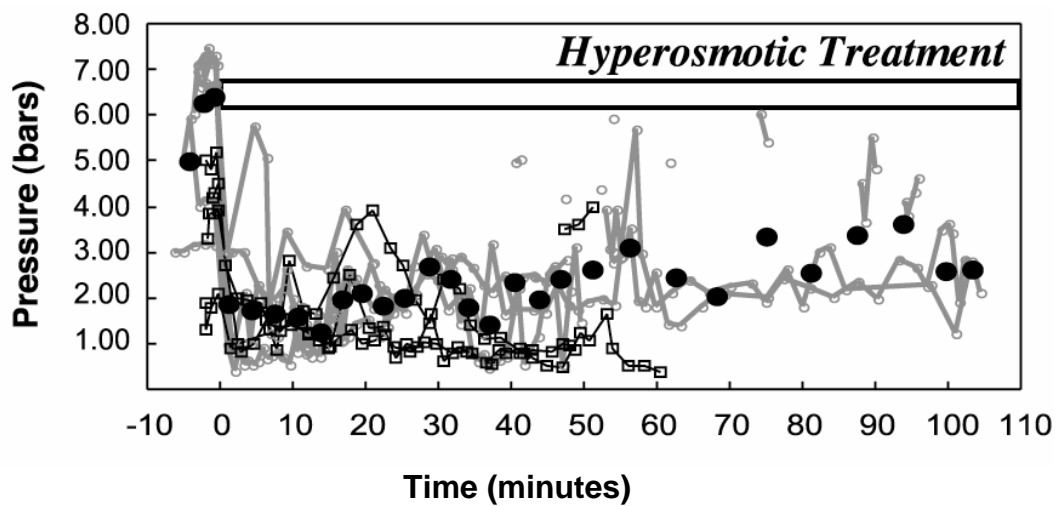


Figure 4.5 - Turgor responses to hyperosmotic treatment of *Achlya bisexualis*. In the experiment, hyperosmotic treatment was applied by adding PYG plus sucrose to hyphae growing in PYG (grey lines) or adding BS plus sucrose to hyphae growing in BS (narrow lines, squares) at time zero as shown. Series of measurements from individual hyphae are shown by continuous lines. The circles are averages for all measurements of PYG-grown cells at intervals of 2.5 or 5.0 minutes. The hyperosmotic environment can be considered approximate to that experienced by hyphae penetrating a substrate.

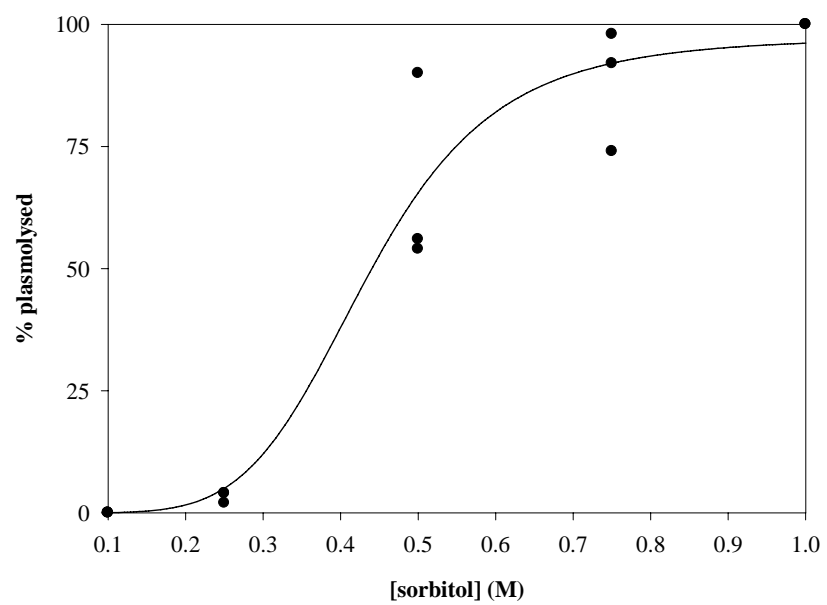


Figure 4.6 – The graph obtained from the plasmolysis of invasive hyphae of *Achlya bisexualis* (plotted using SigmaPlot 2000).

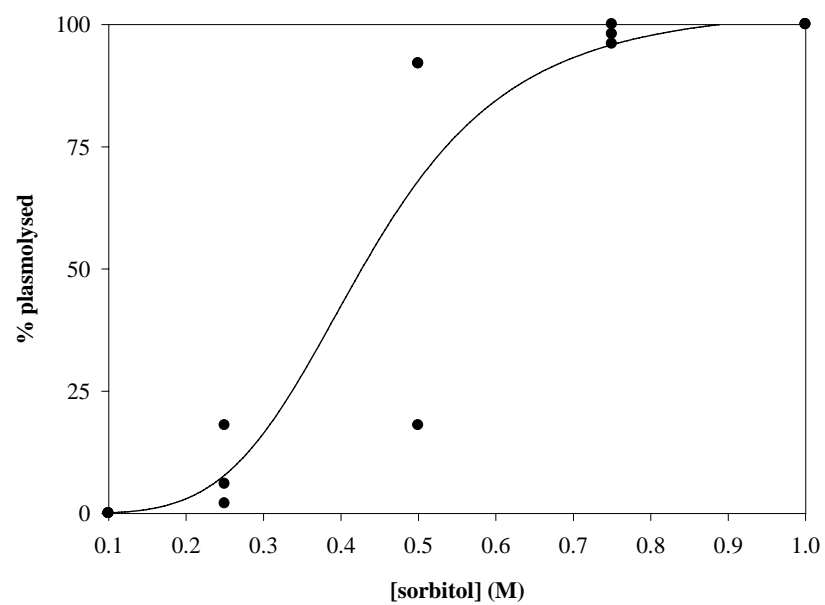


Figure 4.7 – The graph obtained from the plasmolysis of non-invasive hyphae of *Achlya bisexualis* (plotted using SigmaPlot 2000).

Growth type	Average initial pressure (bars)	Average burst pressure (bars)	Average difference (bars)
Invasive	7.80 ± 0.52	10.21 ± 0.85	2.41 ± 0.79
Non-invasive	6.99 ± 1.88	10.75 ± 1.35	3.75 ± 0.76

Table 4.1 – Burst pressure data table. Five replicates of both invasive and non-invasive measurements were carried out resulting in the above averages. The mean increase in pressure required to burst the invasive and non-invasive hyphae were significantly different ($P < 0.05$). Note that due to fact that the offset has not been removed from the initial and burst pressures, these are not directly comparable to the pressure data presented earlier in the thesis.

Discussion

Invasive and non-invasive hyphae experience different environmental conditions. This is due in part to the resistance provided by the substrate, experienced by those cells penetrating the media. The force required for cell extension to occur under these conditions is higher than that required for hyphae growing non-invasively. Traditionally turgor pressure has been considered the driving force behind hyphal extension. An increasing body of evidence suggests that although turgor is important for fungal growth and reproduction, it does not play an essential role in all hyphal species or in all types of cell. In 1999, Money hypothesised that turgor pressure was required for invasive but not non-invasive growth (Money, 1999a). If this were the case then a disparity between the pressures of the invasive and non-invasive cells might be expected.

The results of experiments carried out during the course of this thesis showed the turgor pressure of invasive and the non-invasive hyphae of *Achlya bisexualis* do not differ. This is not surprising given that these data and that of Money (1992) indicate that *A. bisexualis* is not able to turgor regulate. Thus turgor is set by the extracellular osmotic potential and not the substrate solidity. For the theory proposed by Money (1990) to be correct, there needs to be an increased yielding of the tip of the invasive hyphae. The lack of difference in pressure between the invasive and non-invasive hyphae suggests that mechanisms other than increased pressure must be employed to enable the penetration of substrates. Both direct and indirect methods of turgor measurement yielded similar results for the invasive hyphae compared to the non-invasive hyphae.

The pressure values obtained from *A. bisexualis* were comparable with previously reported values for this species of between 7 and 12 bars, measured using vapour pressure deficit osmometry, incipient plasmolysis and pressure probe (Money and Harold, 1993). Other reported values for oomycetes are 7 bars for *Aphanomyces euteiches* (Hoch and Mitchell 1973), 12 bars for *Phytophthora cinnamomi*, 25 bars for *Phytophthora cryptogea* (Woods and Duniway, 1986) and 3-6 bars for *Saprolegnia ferax* (Kaminskyj et al., 1992) all measured using either vapour pressure deficit osmometry or incipient plasmolysis. Direct measurements have also been made on

this latter species giving a value of 4 bars (Money and Harold, 1993). The variability is likely caused by interspecific differences and the variation in the methodologies employed.

The differential between the initial pressures and those required to burst the invasive hyphae were found to be significantly lower than that of those growing non-invasively. This fact suggests there is a difference in the wall tensile strength. This is a possible mechanism by which invasively growing cells may combat the issue of the resistance of the surrounding substrate. If the wall at the apex of the cell is more plastic then less force would be required to drive expansion. It is possible that the exocytotic mechanisms and the action of polymer loosening enzymes may weaken the wall (Money and Hill, 1997). These processes are thought to occur predominantly at the apex and may cause a region of structural weakness (Wessels, 1986; Barnicki-Garcia and Lippman, 1972; Cosgrove, 1987, 2000).

Turgor regulation was not observed in *A. bisexualis* confirming the generally accepted theory that oomycetes are incapable of regulating their internal water or solute potential. In such organisms it could be expected that the invasive and non-invasive hyphae would have the same turgor pressure assuming the water potentials of the substrate and surrounding solution were the same. Thus these organisms should employ alternative mechanisms for invasive and non-invasive growth. Data obtained by Lew et al (2004) demonstrated a difference in the response of *Neurospora crassa* to osmotic challenge compared to that observed in *A. bisexualis*. Upon hyperosmotic treatment the turgor of the hyphae of *N. crassa* declined to near zero then recovered over a period of 100 minutes indicating that turgor regulation did occur. The mechanisms involved were hypothesised to be related to the accumulation of osmolytes and ion transport and to reflect the different phylogenetic lineages of the oomycetes and fungi.

The ability to turgor regulate is likely to be related to the natural habitat of these organisms. Those of terrestrial origins such as *N. crassa* would be more likely to encounter hyperosmotic conditions than aquatic species such as *A. bisexualis*. As hyperosmotic conditions can be likened to those encountered by organisms growing invasively, species that are able to turgor regulate may employ different mechanisms

of invasion to those that cannot. Further work is required to investigate whether the invasive and non-invasive hyphae of species that are able to turgor regulate have variable turgor pressures according to the mode of growth being employed.

CHAPTER 5

Mycelial morphology

Quantifying the characteristics of fungal mycelia

In order to objectively compare inter- and intraspecific variations in mycelial morphology quantitative measures must be made. Early studies could only describe the parameters of fungal systems qualitatively, making comparisons between analyses difficult. A variety of techniques have been developed to quantify the determinants of a hyphal colony. These include the quantification of:

- Radial extension; this is easy to obtain and is frequently used to interpret the health of a colony. It may however be a mechanism of “escape” for the organism and does not directly address branching patterns.
- Biomass; this has historically been the most commonly employed technique and is a measure of how much of the organism is present in a sample. However it provides little indication of the activity of the organism. Similar biomass results can be obtained from organisms employing different growth strategies.
- Hyphal surface area cover; this can be estimated by a simple count of the number of pixels that comprise a captured image. This technique provides important information regarding the total area actually occupied by mycelia.
- Density; this is another measure of the space filling capacity of a system. Again important functional details can be lost by “averaging” the heterogenicities of the system.
- Fractal dimension; this is currently the most effective way to quantify the extent to which a colony permeates space in relation to the extent of the

system. Like many irregular structure in biology, mycelia are approximately fractal therefore fractal geometry is used to quantify the characteristics of a colony (Hitchcock et al, 1996; Richardson, 1961; Morse et al, 1985; Boddy et al, 1999; Patankar, 1980). The two parameters often investigated are border fractal and mass fractal. These are respective measures of the regularity of the hyphal border and the area within this border occupied by mycelia (Donnelly et al, 1995; Obert et al, 1990). The results generated can be used to calculate the total mycelial area and therefore the average radial extension.

Fractal geometry of hyphal systems

Fractal geometry helps describe structures which don't conform to Euclidean geometry which measures parameters in whole number dimensions of 1 (straight lines), 2 (flat surfaces) and 3 (volumes) (Schmid and Harper, 1985). Ideal fractals are self-similar over an infinite range of scales whereas natural structures are generally self-similar over a finite range of scales (Hastings and Sugihara, 1993). Despite this, they are better described using fractal geometry rather than regular profiles. The images that can be generated from these structures are commonly termed random fractals. The use of fractal geometry enables the quantitative description of complex shapes. Such objects are examined at an increasingly closer magnification and as the image is enlarged more irregularities become apparent.

Several recent studies have used fractal dimensions to describe fungal colonies (Boddy et al, 1999; Richardson, 1961; Morse et al, 1985; Lundy et al, 2001; Patankar, 1993). Mycelia are confined within finite planes and volumes, but they are effectively infinite when viewed on an infinite scale. The ability of this method to identify irregularities in a fractal pattern enables the identification of morphological differences not detectable by other analytical methods. It is important to quantify both the space filling capacity of a colony at mycelial margins and within those boundaries (Hitchcock et al, 1996; Obert et al, 1990). Border and mass fractal analysis are appropriate for the investigation of these variables. The mass fractal is a descriptor of the area covered by the mycelium within the minimum perimeter that can contain the entire colony. The border fractal value is achieved by comparing the sum of all the perimeters within the system with the minimum perimeter. Several

methods have been developed to calculate mass and border fractals. The first fractal study of mycelia in soil, like that of several studies of hyphal organisms on agar, employed the concentric circle method (also known as the two point density correlation function method) (Bolton and Boddy, 1993). This technique is somewhat inadequate in that it excludes the growth margin from the analysis. The subsequently developed ‘box count’ method (Obert et al, 1990) enables the estimation of both the border and mass fractals and has been employed in the analysis of hyphal systems in soil (Donnelly et al, 1995; Abdalla and Boddy, 1996; Donnelly and Boddy, 1997a,b; Wells et al, 1997; Donnelly and Boddy, 1998) and agar (Mihail et al, 1994, 1995; Lundy et al, 2001). This is the currently the most commonly employed fractal technique as it involves the analysis of the entire system including the mycelial margin. “Box counting” involves overlaying the mycelial image with grids of varying dimensions. For each lattice size the number of boxes intersecting the image is counted. Fractal images obey the ‘power law relation’ over a range of scales:

$$N(s) = cs^{-D_B} \quad \text{Equation 5.1}$$

$N(s)$ represents the total number of boxes of side length s that intersect the image and c is a constant. The box counting dimension is defined by D_B and is estimated as a negative value because it is the gradient of a regression line through the linear part of the plot of $\log N(s)$ against $\log s$, for a sequence of scales s (Hastings and Sugihara, 1993). D_{BS} (border fractal) is obtained by the regression of $\log N_{\text{border}}$ against $\log s$. Because the boxes at the border are not entirely filled with the image, the area of the mycelium is progressively overestimated by larger box sizes. In order to obtain an accurate estimate of D_{BM} (mass fractal), $\log [N(s) - \frac{1}{2} N_{\text{border}}(s)]$ is regressed against $\log s$ (Boddy et al, 1999). The resulting values are numerical representations of the structural characteristics of the hyphal colony and provide a very effective method of comparison of morphological information.

The fractal dimensions of a colony can be used to describe two factors regarding hyphal growth. Firstly, the morphogenesis of the mycelia with relation to the extracellular substrate conditions in agar can be mathematically defined (Patankar et al, 1993; Regalado et al, 1996, Jones et al, 1995). The reaction-diffusion model of growth suggests that the fractal nature of mycelia is generated in response to

intracellular activators and inhibitors as well as extracellular interaction (Regalado et al, 1996). This model allows for variation of the extracellular substrate thereby enabling predictions to be made regarding mycelial form in heterogeneous environments. It is, however, based upon a situation in which hyphal growth radiates from a point of inoculum such as a spore. This is very rarely the case in natural environments. The second use for fractal dimensions is the quantification of interspecific differences in mycelial morphology which can be related to natural habitat (Donnelly et al, 1995; Mihail et al, 1995). It has been found that some species produce mass and border fractal systems whereas others generate only border fractal patterns (Donnelly et al, 1995). This is most likely due to interspecific differences in growth strategy. For example non-unit restricted hyphal organisms may be insensitive to the effects of a heterogeneous environment due to their intracellular nutrient supply and the ability to effectively translocate these nutrients within the mycelia (Boddy, 1999; Wells et al, 1990; 1995). This would inevitably affect the mass fractal dimension. Homogenous environments, often used in controlled laboratory based experiments are much easier to analyse than naturally occurring heterogeneous environments. By minimising heterogeneity, the responses to specific environmental conditions can be examined. Once mycelial growth responses to certain environmental stimuli are understood, the mechanisms of extension and invasion employed by those organisms may be clarified.

Some important factors have to be taken into account when considering the results arising from the fractal analysis of a fungal colony. It must be accepted that all descriptors of hyphal systems are valid but unrelated. Fractal dimensions may correlate to measurements such as hyphal surface area but this does not provide evidence for a causal link between the two, just as hyphal surface area cannot be directly correlated to radial extension. Consequently a range of descriptors need to be employed for the correct analysis of the morphology of a system. Despite the effectiveness of fractal geometry as an analytical technique, observation can play a key role in intra- and interspecific comparisons. For example, mycelia with similar fractal dimensions may display radically different branching patterns and non-unit restricted colonies will employ a variety of growth strategies dependant on the substrates that are encountered. The response of mycelia encountering new resources often involves the relocation of biomass and is dependant on a variety of factors

including the species involved, quantity and quality of the resource, and the nutrient status of the surrounding substrate. Variations in nutrient status, competition and other environmental factors may result in morphological disparity within the colony (Boddy, 1999). Often patches are formed in response to the physiological demands of the colonization and assimilation of a new resource (Muller et al, 2000). These ultrastructural anomalies are not investigated through the ‘averaging’ of the system. This fact means that fractal geometry can only be used to describe either naturally or artificially unit-restricted organisms and cannot describe finer morphological differences between colonies. Despite these issues regarding fractal geometry, it remains the most effective method of quantitative analytical study of the morphology of hyphal organisms. The further development of imaging technologies will allow a more detailed analysis of fractal structures.

As the morphology of cells is determined in part by the environmental conditions they encounter, invasive and non-invasive mycelia may display disparate morphological and physiological characteristics. The physical and biochemical features of a substrate affect the hyphae at a cellular level which is evident in the analysis of the system as a whole. In order to determine the effect of agar concentration on the mycelial morphology, fractal analysis was employed. This quantified the effect of the solidity of the substrate on the patterns of growth exhibited by a variety of hyphal species. The hyphal species that were analysed during the course of this thesis were *Achlya bisexualis*, *Neurospora crassa* and *Mortierella wolfii*. *A. bisexualis* is a fresh water aquatic species of oomycete. This was used as an example of an organism whose natural habitat typically does not require penetration of solid substrates. *N. crassa* is a terrestrial organism whose natural habitat does require penetrative growth. *M. wolfii* is a zygomycete that is known to exist in decaying plant material. It is a pathogen of cattle and has been cultured from blood as well as oxygen rich organs such as the placenta, brain and lungs. This organism is able to employ non-invasive growth in fluid environments and persistent invasive growth in more solid organic matter. The ability of this organism to employ a variety of modes of growth makes it ideal for comparative fractal analysis.

Materials and methods

Materials and methods were as described in Chapter 2.

Results

This series of experiments only examined the differences between mycelia growing in media containing 1% - 8% agar. Non-invasive samples (i.e. in 0% agar) were attempted but unfortunately due to technical difficulties regarding imaging the mycelia that developed under these conditions, they could not be compared to those growing invasively.

Fractal geometry of *Achlya bisexualis*

Hyphae of *Achlya bisexualis* were grown on media ranging in concentration from 1% through to 8% agar (w/v). The initial images of the mycelia were obtained using a scanner as the depth of the focal plane enables imaging of three dimensional objects and approximating them to two dimensional images. This facilitates the imaging of both the invasive and non-invasive hyphae of the mycelia. Examples of images obtained by the image capture and processing technique (as described in Chapter 2) are shown in Figure 5.1. Fractal analysis was applied to these colonies. The average border fractal D-value of the colonies on 1% agar was significantly higher than that of those on 2% agar ($P < 0.0001$, $n = 20$). With increasing agar concentration above 2% there was a corresponding increase in border fractal D-values, with values at 5% - 8% significantly higher than at 2% ($P < 0.0001$, $n = 20$). These results are summarised in Figure 5.2. High border fractal D-values are indicative of a high degree of irregularity at the mycelial margin. The average mass fractal D-values increased as the concentration of agar increased. This means that generally, as the media became more difficult to penetrate, the mycelia became more compact with less space occurring between hyphae. These results are summarised in Figure 5.3. The average mass fractal D-value of the colonies on 1% agar is significantly different to those on 7% and 8% agar. And the average mass fractal D-value of the colonies on 4% agar is significantly different to those on 7% agar ($P < 0.0006$, $n = 20$). The mycelial area generally decreased with increasing substrate solidity. These results are summarised in Figure 5.4. The average mycelial area of the colonies on 4% agar is significantly different to those on 1%, 2%, 5%, 6%, 7%, and 8%. The average mycelial area of the colonies on 5% agar is significantly different to those on 1%, 2%, 3%, 6%, 7%, and 8%. The average mycelial area of the colonies on 7% agar is significantly different to

those on 1%, 2%, 3%, 6%, and 8%. The average mycelial area of the colonies on 8% agar is significantly different to those on 1%, 2%, and 3% ($P < 0.0001$, $n = 20$).

Fractal geometry of *Neurospora crassa*

Hyphae of *Neurospora crassa* were grown on media ranging in concentration from 1% through to 8% agar (w/v). Examples of the images obtained by the image capture and processing technique (as described in Chapter 2) are shown in Figure 5.5. The border fractal analysis displayed a general trend of decreasing D-values with increasing agar concentration. These results are summarised in Figure 5.6. The average border fractal D-value of the colonies on 1% agar is significantly different to those on 2%, 3%, 4%, 5%, 6%, 7%, and 8%. The average border fractal D-value of the colonies on 2% agar is significantly different to those on 3%, 5%, 6%, and 7% ($P < 0.001$, $n = 20$). The reduction in D-value indicates a development of order at the growing margin with increasing agar concentration. The results of the mass fractal analysis of these images were inconclusive as some of the average D-values obtained are over the maximum value of 2.0. This effectively means that the mycelium behind the growing margin was too dense to allow meaningful analysis as significant overlapping of hyphae may have been occurring. These results are summarised in Figure 5.7. The effect of media viscosity on mycelial area produced results which are summarised in Figure 5.8. Initially the average mycelial area rises upon increase of agar concentration but then drops before beginning to rise again at 8%. The average mycelial area of the colonies on 1% agar is significantly different to those on 3%, 4%, and 5%. The average mycelial area of the colonies on 7% agar is significantly different to those on 2%, 3%, 4%, 5%, 6%, and 8% ($P < 0.0001$, $n = 20$).

Fractal geometry of *Mortierella wolfii*

Hyphae of *Mortierella wolfii* were grown on media ranging in concentration from 1% through to 8% agar (w/v). Examples of the images obtained by the image capture and processing technique (as described in Chapter 2) are shown in Figure 5.9. The border fractal analysis displayed a general trend of decreasing D-values upon increasing media agar concentration. These results are summarised in Figure 5.10. The average border fractal D-value of the colonies on 1% agar is significantly different to those on

2%, 3%, 4%, 5%, 6%, 7%, and 8%. The average border fractal D-value of the colonies on 2% agar is significantly different to those on 5%, 6%, 7%, and 8%. The average border fractal D-value of the colonies on 3% agar is significantly different to those on 4%, 5%, 6%, 7%, and 8%. The average border fractal D-value of the colonies on 5% agar is significantly different to those on 6%. The average border fractal D-value of the colonies on 6% agar is significantly different to those on 7% and 8% ($P < 0.0001$, $n = 20$). Again the reduction in the D-value indicates a development of order at the growing margin upon increasing agar concentration. The mass fractal analysis indicates that the density of the mycelia did not vary significantly upon increased substrate solidity. Most of the average D-values for this analysis were over the value of 2.0 therefore no valid conclusions can be drawn from these results as again, the mycelium behind the growing margin was too dense to apply fractal geometry to. These results are summarised in Figure 5.11. A decrease in the average mycelial area upon increasing agar concentration was observed. These results are summarised in Figure 5.12. The average mycelial area of the colonies on 1% agar is significantly different to those on 3%, 4%, 5%, 6%, 7%, and 8%. The average mycelial area of the colonies on 2% agar is significantly different to those on 4%, 5%, 6%, 7%, and 8%. The average mycelial area of the colonies on 3% agar is significantly different to those on 5%, 6%, 7%, and 8%. The average mycelial area of the colonies on 4% agar is significantly different to those on 6%, 7%, and 8%. The average mycelial area of the colonies on 5% agar is significantly different to those on 8% ($P < 0.0001$, $n = 20$).

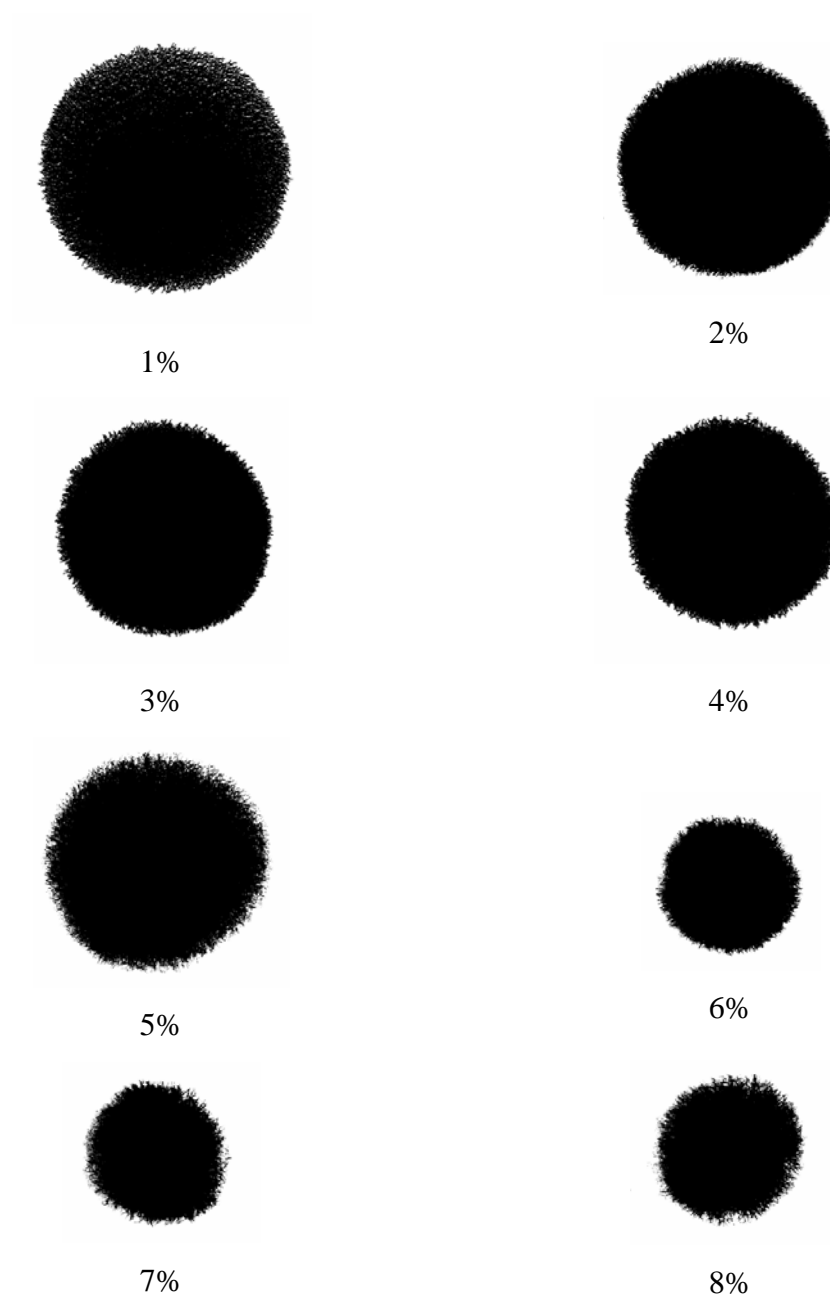


Figure 5.1 – Processed images of *Achlya bisexualis* generated from colonies grown on media containing different concentrations of agar.

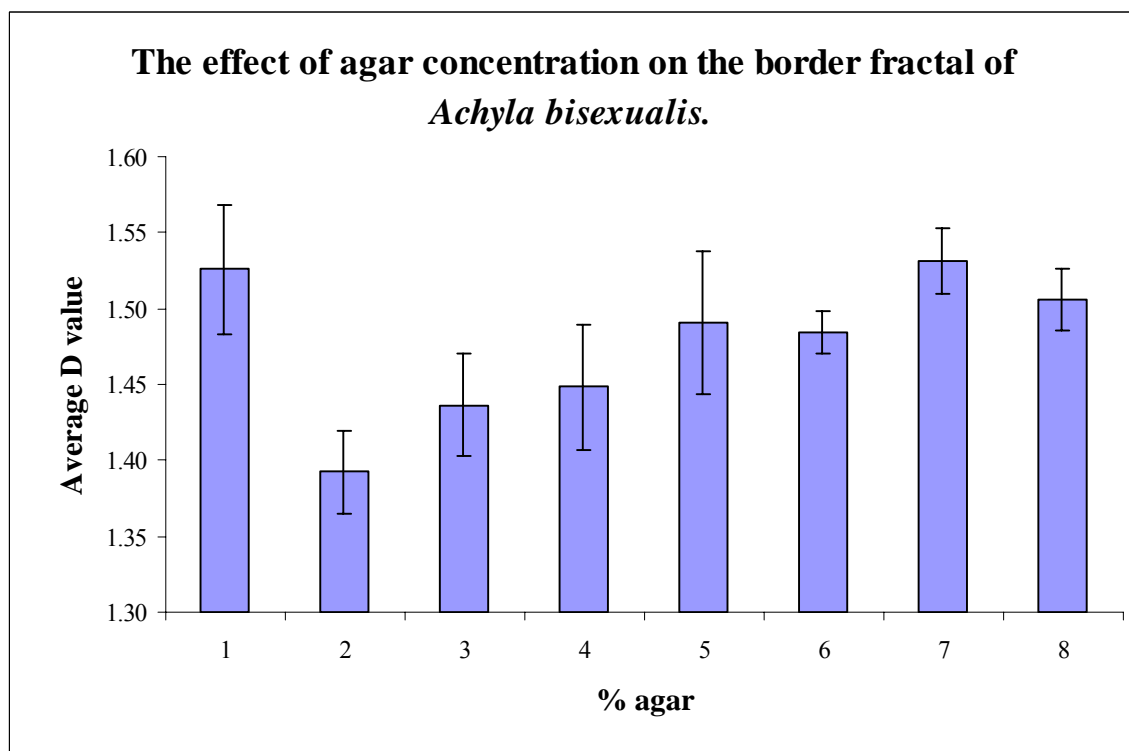


Figure 5.2 - High border fractal D-values of *Achlya bisexualis* colonies are indicative of increased irregularity at the mycelial margin. Statistical analysis revealed that concentrations 1% \neq 2%, 3%, 4%; 2% \neq 5%, 6%, 7%, 8%; 3% \neq 7% and 4% \neq 7% ($P < 0.0001$).

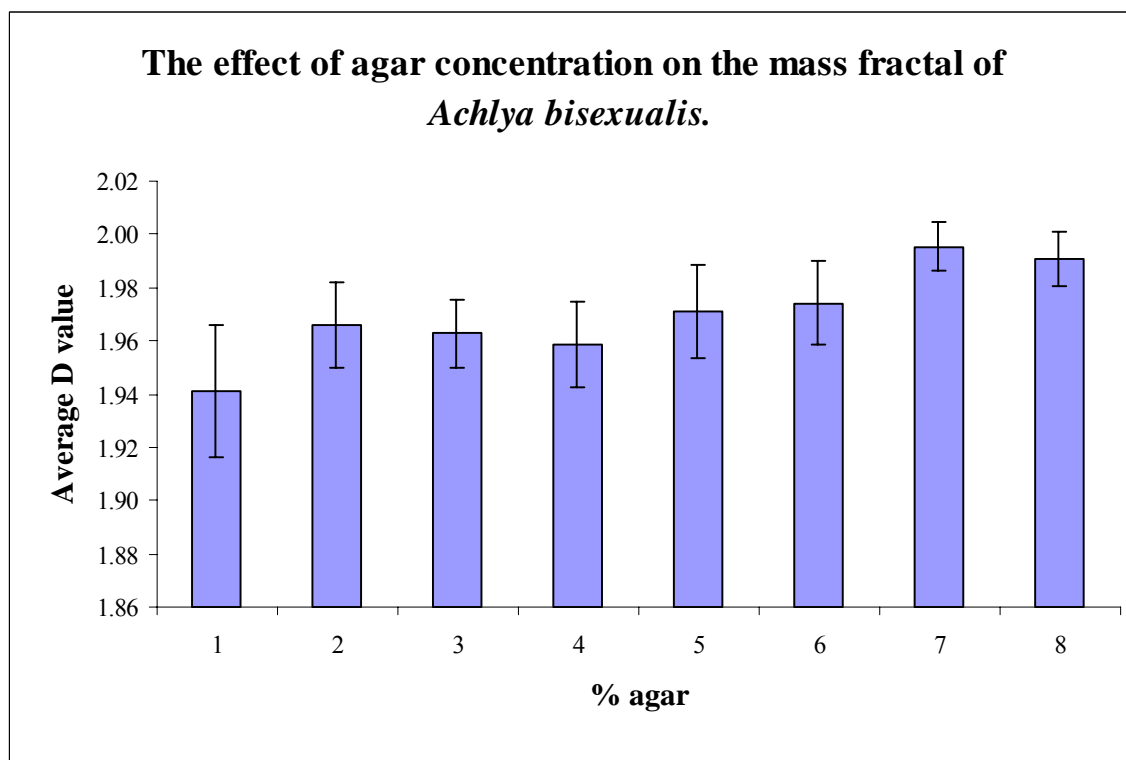


Figure 5.3 - As the media became harder to penetrate, the mycelia of *Achlya bisexualis* became more compact with less space occurring between hyphae. Statistical analysis revealed that concentrations 1% \neq 7%, 8% and 4% \neq 7% ($P < 0.0006$, $n = 20$).

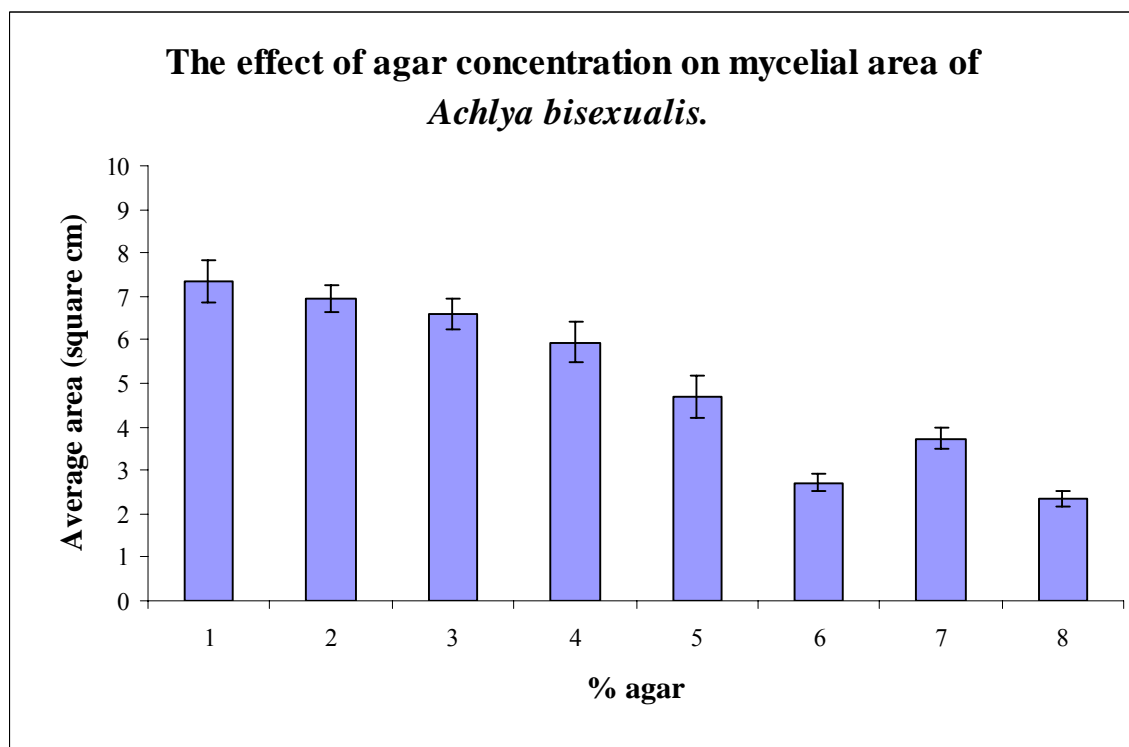


Figure 5.4 - The mycelial area of *Achlya bisexualis* colonies generally decreased as agar concentration increased. Statistical analysis revealed that concentrations 4% \neq 1%, 2%, 5%, 6%, 7%, 8%; 5% \neq 1%, 2%, 3%, 6%, 7%, 8%; 7% \neq 1%, 2%, 3%, 6%, 8% and 8% \neq 1%, 2%, 3% ($P < 0.0001$, $n = 20$).

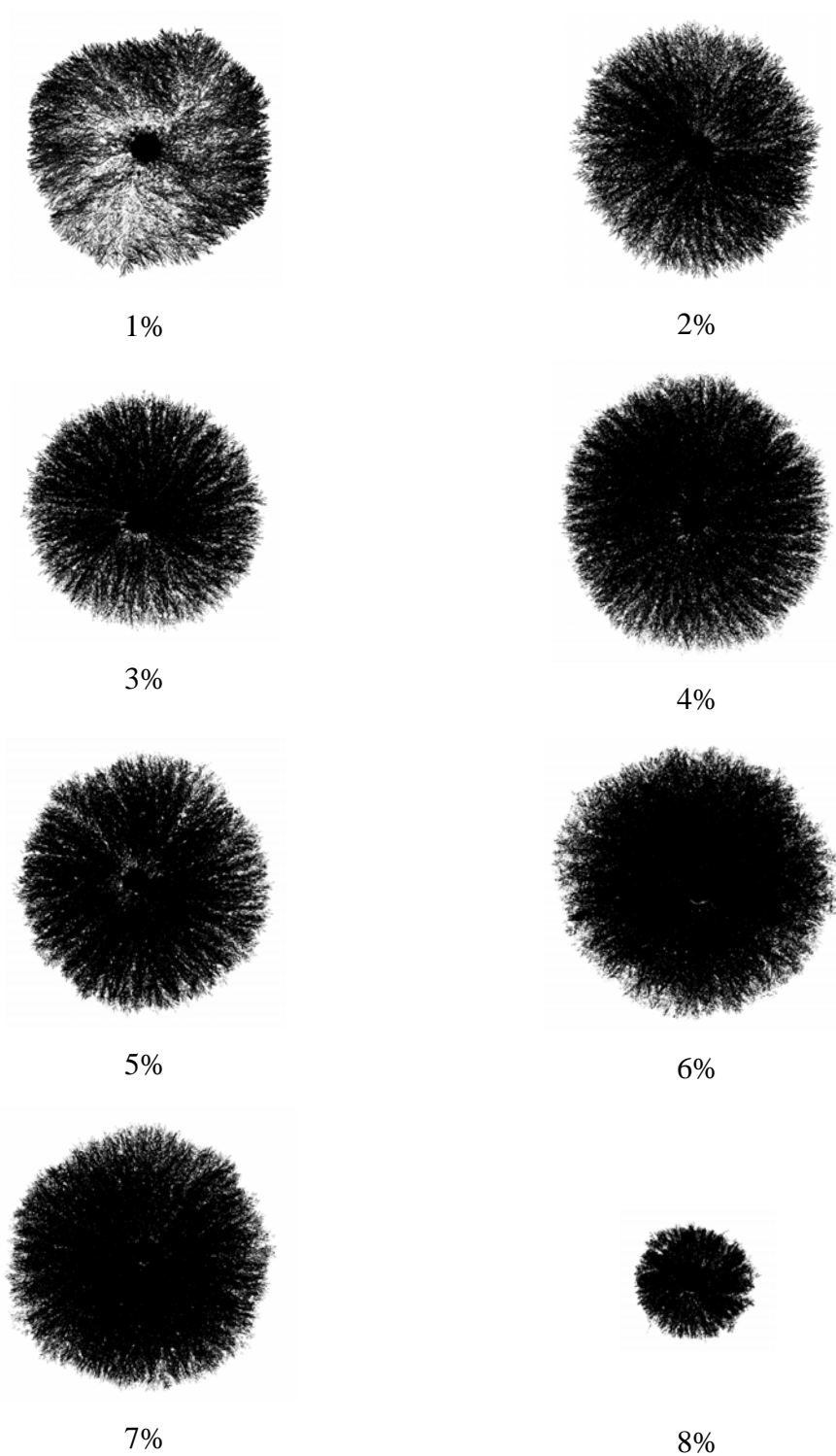


Figure 5.5 – Processed images of *Neurospora crassa* generated from colonies grown on media containing different concentrations of agar.

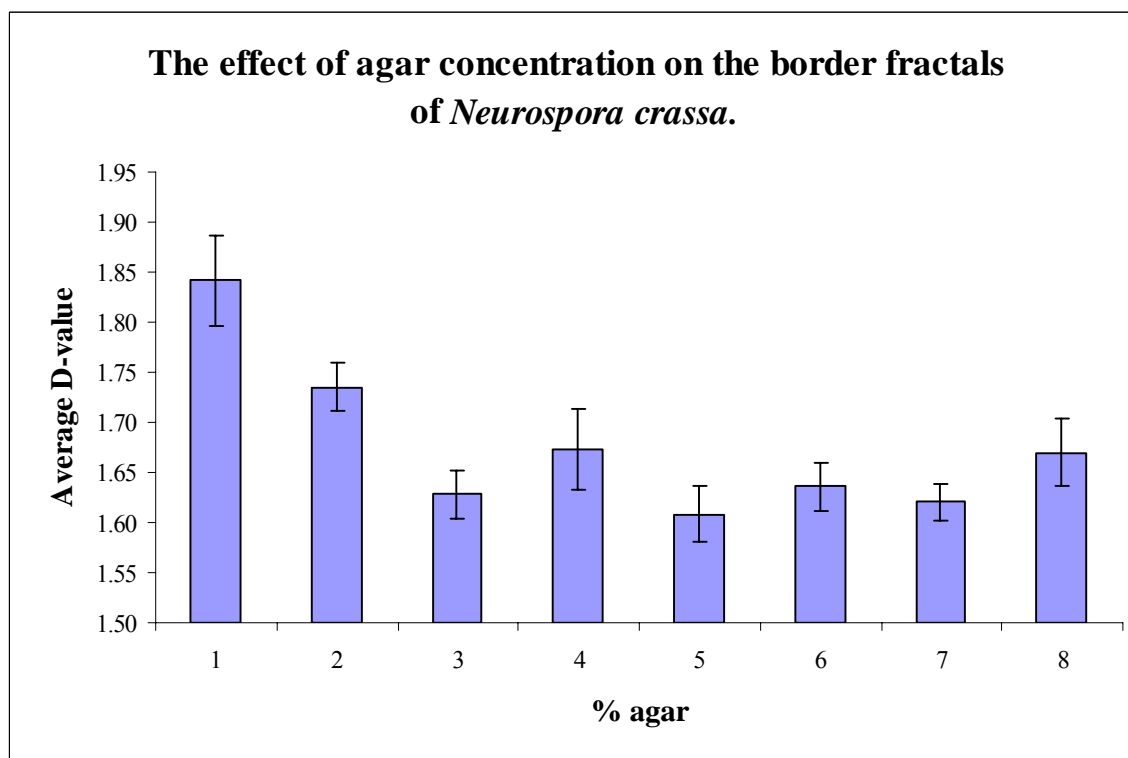


Figure 5.6 - The border fractal analysis of *Neurospora crassa* colonies displayed a general trend of decreasing D-values upon increasing agar concentration. Statistical analysis revealed that concentrations 1% \neq 2%, 3%, 4%, 5%, 6%, 7%, 8% and 2% \neq 3%, 5%, 6%, 7% ($P < 0.001$, $n = 20$).

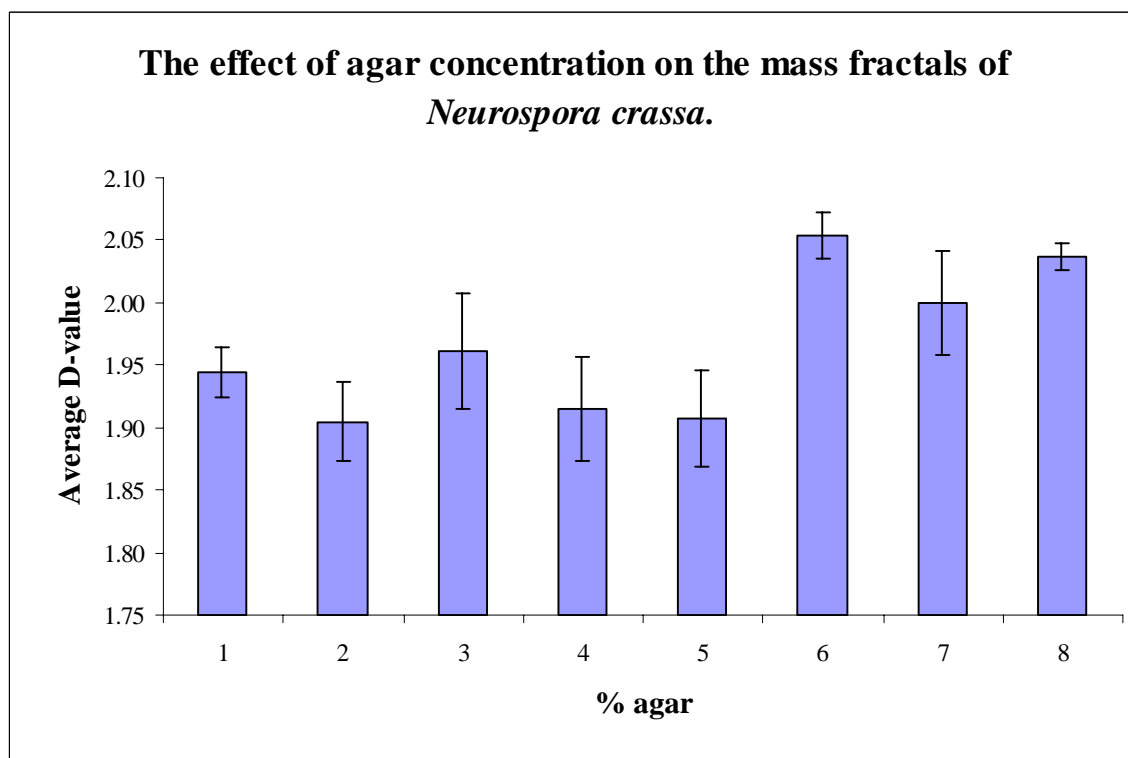


Figure 5.7 - The results of the mass fractal analysis of the images of *Neurospora crassa* colonies were inconclusive as some of the average D-values obtained were over the maximum value of 2.0. This means that the mycelium behind the growing margin was too dense to allow meaningful analysis.

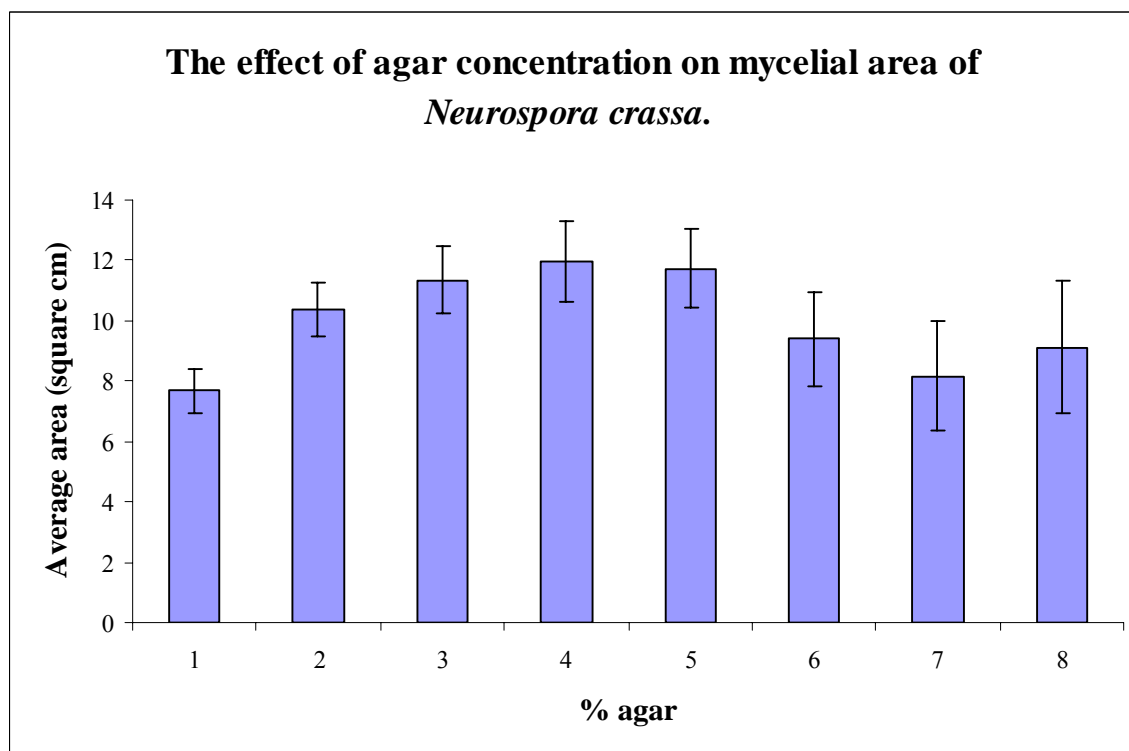


Figure 5.8 - The average mycelial area of *Neurospora crassa* colonies initially rises upon increase of agar concentration but then drops before beginning to rise again at an agar concentration of 8%. Statistical analysis revealed that concentrations 1% \neq 3%, 4%, 5% and 7% \neq 2%, 3%, 4%, 5%, 6%, 8% ($P < 0.0001$, $n = 20$).

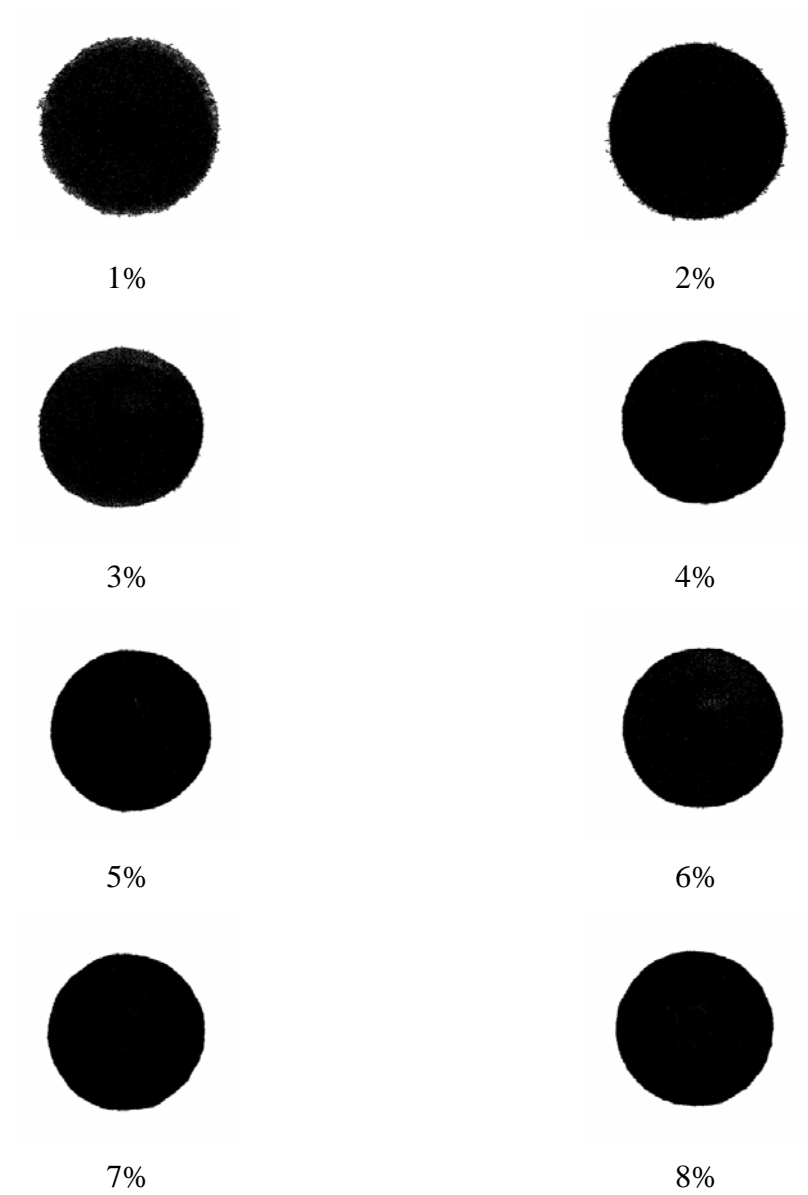


Figure 5.9 – Processed images of *Mortierella wolfii* generated from colonies grown on media containing different concentrations of agar.

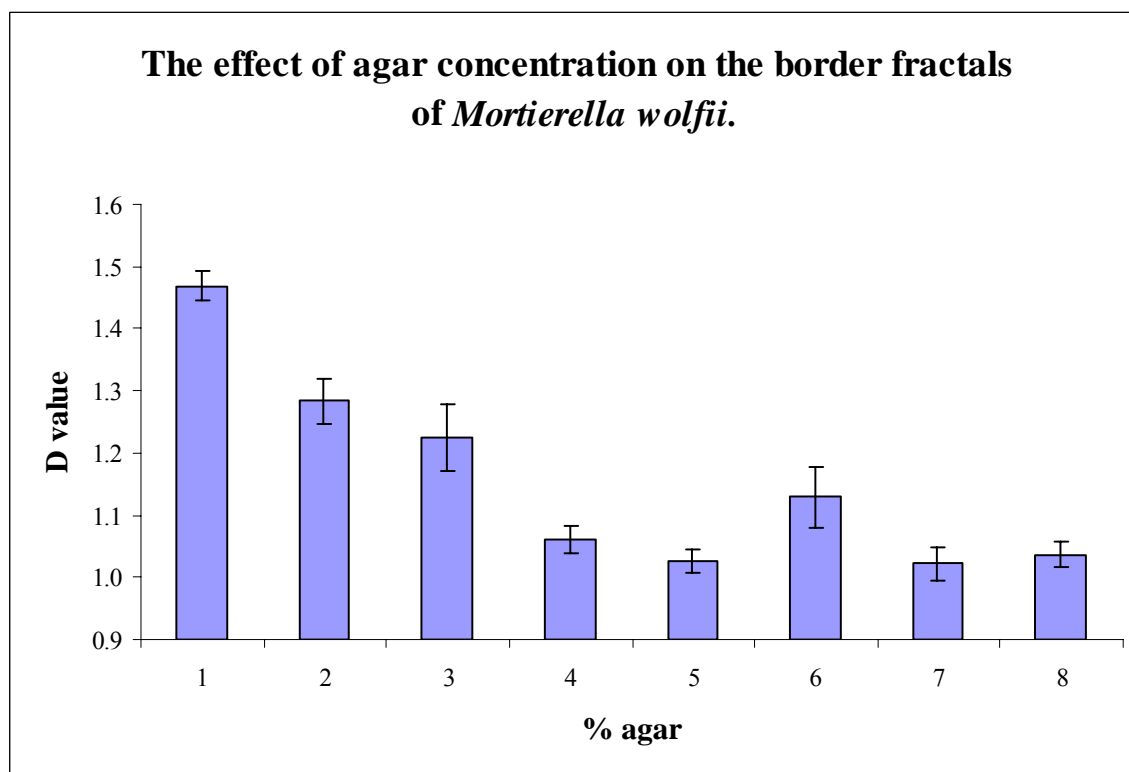


Figure 5.10 - The border fractal analysis of *Mortierella wolfii* colonies displayed a general trend of decreasing D-values upon increasing media agar concentration. Statistical analysis revealed that concentrations 1% \neq 2%, 3%, 4%, 5%, 6%, 7%, 8%; 2% \neq 5%, 6%, 7%, 8%; 3% \neq 4%, 5%, 6%, 7%, 8%; 5% \neq 6% and 6% \neq 7%, 8% ($P < 0.0001$, $n = 20$).

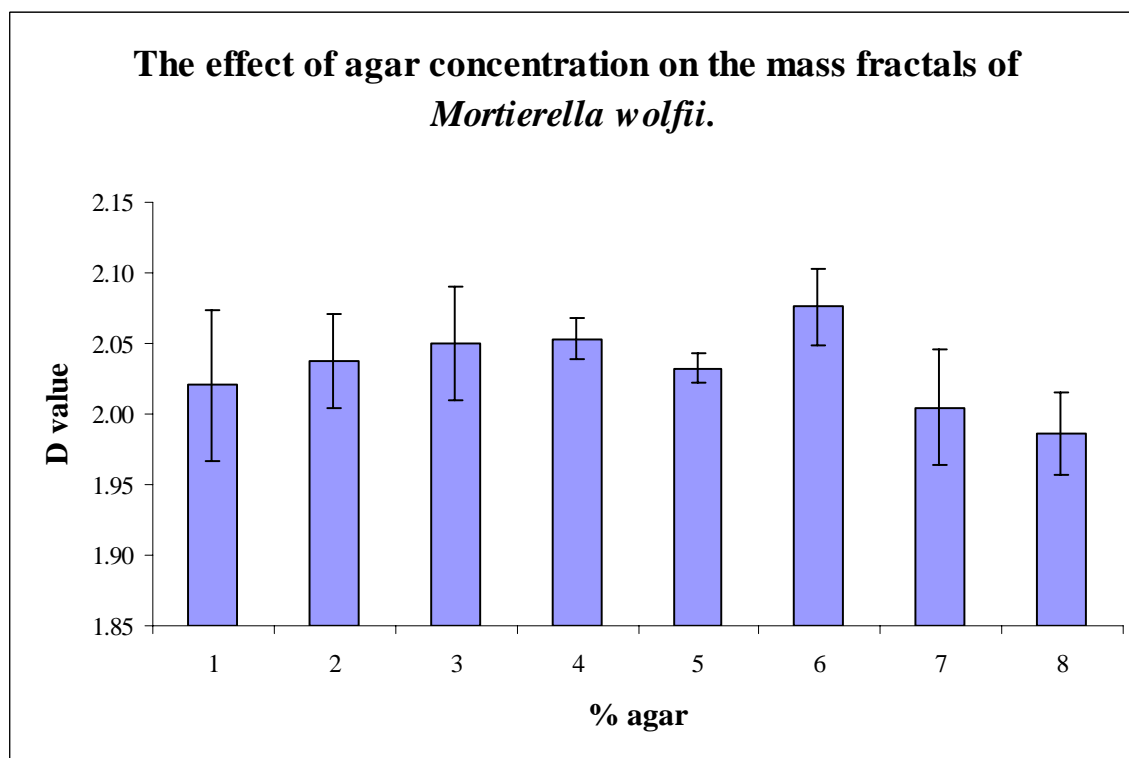


Figure 5.11 - The results of the mass fractal analysis of the images of *Mortierella wolfii* colonies were inconclusive as some of the average D-values obtained were over the maximum value of 2.0. This means that the mycelium behind the growing margin was too dense to allow meaningful analysis.

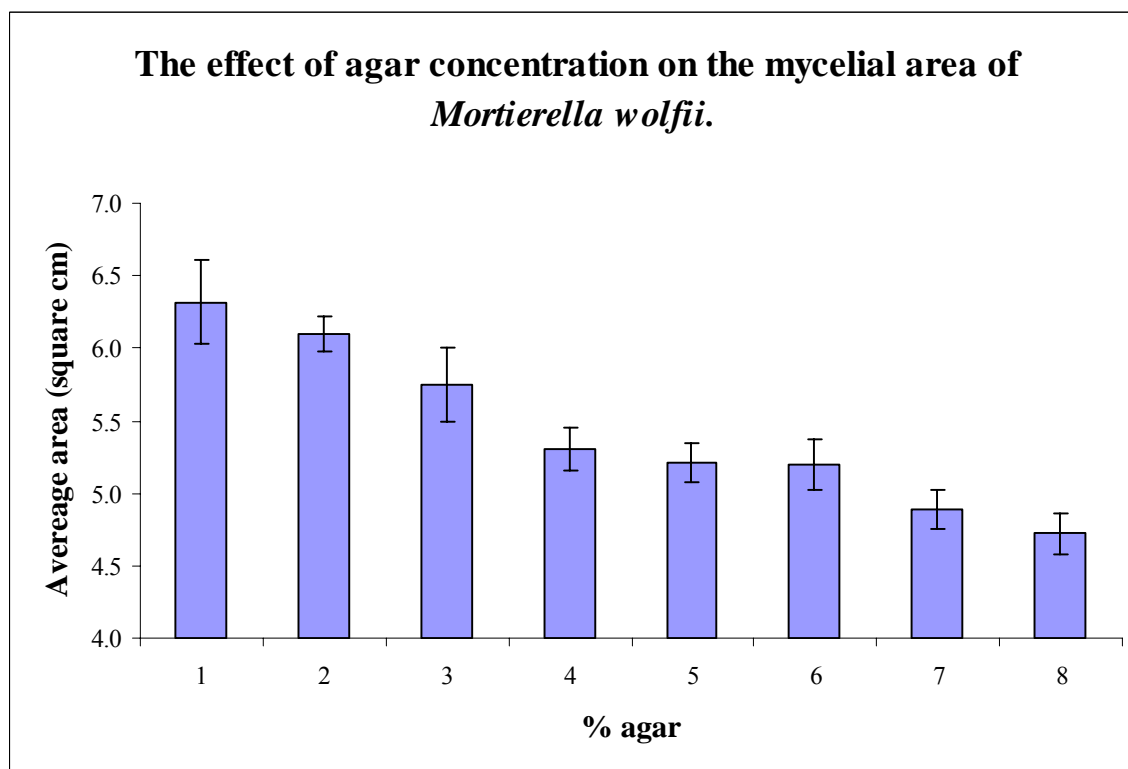


Figure 5.12 - A decrease in the average mycelial area of *Mortierella wolfii* colonies upon increasing agar concentration was observed. Statistical analysis revealed that concentrations 1% \neq 3%, 4%, 5%, 6%, 7%, 8%; 2% \neq 4%, 5%, 6%, 7%, 8%; 3% \neq 5%, 6%, 7%, 8%; 4% \neq 6%, 7%, 8%; 5% \neq 8%; and ($P < 0.0001$, $n = 20$).

Discussion

The fractal analyses described indicate that variation of agar concentration affects the growth in the three species examined. This suggests that the pattern of growth is influenced by the solidity of the substrate the organism is growing in.

Achlya bisexualis is a freshwater, aquatic species. The average D-values of the border fractals of *A. bisexualis* were initially high at 1% agar dropping at 2%, gradually rising until they reached approximately the same value as that of the 1% agar, at an agar concentration of 7%. This indicates that in 1% agar the system has a relatively irregular margin which became more ordered in 2% agar. In 3% - 8% agar, the margin became progressively more ordered. The average D-values for the mass fractal gradually increased as the concentration of agar in the media increased. This indicates that the mycelia grew closer together as the media got harder. As this occurred, the radial extension of the systems decreased. This suggests that the growth that typically occurred in a radial manner began to be diverted towards increasing branching frequencies within reduced mycelial margins. Alternatively it may also indicate a reduction in growth rate.

Neurospora crassa is a terrestrial fungal species whose natural habitat is commonly soil. This may be reflected in the patterns of growth observed. The average border fractal D-value decreased as agar concentration increased indicating that the margin became more regular as the solidity of the substrate increased. The average mass fractal D-value did not display a strong trend however many of the values were over the maximum D-value of 2.0. This indicates that the mycelium behind the growing margin was too dense to allow meaningful analysis to be carried out as significant overlapping of hyphae may have been occurring. This may be a function of the high nutrient status of the media. The highest average fractal area occurred at an agar concentration of 4%. The disparity between these results and that of *A. bisexualis* may be a likely function of the natural habitats of the species and the resultant difference in growth strategies.

Mortierella wolfii is a pathogenic zygomycete with the capacity to invade a variety of bovine tissues from blood to solid organs such as the brain, lungs and placenta.

Infection of cattle is thought to occur upon exposure to mulch containing the fungus. The diversity of the resources utilised by this fungus make it an interesting candidate for analysis. The average border fractal D-values decreased as agar concentration increased indicating that the margin became more regular as the solidity of the substrate increased as it did with *N. crassa*. The mass fractal analysis yielded many D-values over 2.0 indicating that the mycelium behind the growing margin was too dense to allow meaningful analysis to be carried out as significant overlapping of hyphae may have been occurring. The fractal area for this species decreased as the as the concentration of agar increased.

Differences in mycelial morphology were evident between the fungal species examined. The pattern of mycelial growth was altered by the agar concentration of the media, the extent of which was generally dependant on the species. In the non-aquatic species there was a trend for more regular mycelial edges with increasing media hardness. In the aquatic oomycete there was an initial decrease in the transition from 1% to 2% agar and then a gradual increase in D-value indicative of a move towards the original, less ordered edge as the agar concentration was increased to 8%. From the analysis carried out it there appears a general trend of decreasing mycelial area upon increasing agar concentration. The responses of the mycelia varied between species with regard to border fractal. The mass fractals results are difficult to compare due to the high proportion of results exceeding the maximum D-value of 2.0.

It should be noted that interspecies comparison of the results are tempered by the fact that the techniques of culturing and image processing employed for each species were unique. *M. wolfii* for example, was cultured on MEA and *A. bisexualis* and *N. crassa* were cultured on PYG. A media specific effect cannot be ruled out without further investigation. In addition to this, different threshold levels were set during the image processing and the cultures were incubated over different periods of time. It was observed during the course of the experiments that fractal plates incubated on different days displayed some morphological variation. As these differences were not significant no further analysis was carried out however this suggests that even slight variations in incubation conditions can have an effect on the results obtained. In addition to this species that develop as mass fractal systems have been shown to change over time. Boddy et al (1999) showed that the low mass fractal values of

Phallus impudicus increased with time and radial extension. *Phanerochaete velutina* initially had high mass fractal values which later decreased. These effects were attributed to a change branching frequency during the development of the colony. It was suggested that more regular branching leads to higher mass fractals. A more radial development as observed in *P. velutina* results in development of cord aggregates behind foraging front as mycelia extends therefore causing a decrease in mass fractal (Bolton and Boddy, 1993).

The mycelial patterns observed are also likely to be associated with extension rate. There is often an inverse relationship between the mass fractal dimension and extension rate. This is a function of contrasting foraging strategies (Donnelly et al, 1995). The establishment of contrasting interspecific mycelial patterns and variant growth rates has been attributed to distinct strategies for the capture of resources which may be subject to variable spatial and temporal distribution (Dowson, 1988a, 1989b). Dense systems with a low border fractal and a high mass fractal employ an effective strategy for the capture of abundant, less dispersed resources. More diffuse systems with a high border fractal and a low mass fractal are more effective at capturing widely dispersed resources. The results of the fractal analysis of *A. bisexualis* suggest that the typical morphology of the organism is such that it is able to grow in media with fairly dispersed resources. Its natural habitat is an aqueous environment but it has been shown to be able to grow on much more solid substrates, with a change in morphology to suit. *N. crassa* has a mycelium that is more diffuse than that of *A. bisexualis*. This may be a function of its natural habitat and the fact that the resources in soil are typically more widely dispersed. *M. wolfii* produces far more dense colonies which may be due to the fact that it naturally occurs in organic matter of high nutrient status. The use of fractal geometry has helped enhance the understanding of growth strategies used by these three species in media containing different concentrations of agar.

There are problems associated with the application of fractal geometry. These include that fact that different growth strategies such as branching pattern can result in similar fractal dimensions. Incongruities within the mycelia can be “averaged” out of the system. As there were no such structures in any of the experiments carried out during the course of this thesis no further analysis or description was required but under more

heterogeneous conditions this may be an issue. As the experiments examined the development of hyphal species in sterile, homogenous media the results cannot be directly compared to the natural environment as these are typically non-sterile, heterogeneous. Therefore the issue of ecological significance must be raised. As the complexities of natural environments are difficult to predict and analyse, experiments carried out under laboratory conditions can provide some useful comparative data concerning general concepts though these may not be directly applicable to natural systems.

CHAPTER 6

Conclusions

Most walled cells generate hydrostatic pressure, or turgor, greater than that of the atmosphere. The pressure results from the influx of water due to the difference in solute concentration between the cytoplasm and the external media. The plasma membrane presses against the cell wall as the cytoplasm swells. The wall is therefore subjected to tension. Historically it has been accepted that this turgor pressure plays an essential role in hyphal extension. This model involves the cytoplasm swelling as turgor develops. The plasma membrane presses against the inner surface of the cell wall. The tensile strength of the wall increases. The cell wall at the apical tip of the hypha loosens, the cell wall polymers relaxing allowing the pressure provided by the turgid cytoplasm to drive extension. There are problems with this theory of turgor pressure driven growth. These include the fact that hyphal extension has been shown to occur even when the hydrostatic pressure was so low that it was unable to be measured. There is also a lack of authoritative testing of and supporting evidence for this theory.

The alternative theory of amoeboid movement likens hyphal extension to the movement of tube dwelling amoeba. This involves the cytoplasm at the apical tip being protruded like a pseudopodium. After the pseudopodium is extended the protrusion adheres to the substrate by means of intracellular proteins similar to integrins. A traction force must then be exerted bringing forward the bulk of the cytoplasm towards the pseudopodium. Unlike true amoeboid movement hyphal extension would not require any translocation of the sub-apical cytoplasm but supplementation of the pre-existing cytoplasm. The cell wall components are then excreted by the means of vesicles and the wall is assembled on the cell surface. The amoeboid theory of growth does not account for the fact that upon loss of turgor, hyphae lose their ability to penetrate substrates, and the ability to form aerial structures and zoospores. This means that turgor must be maintained in at least some structures for successful growth to occur (Pickett-Heaps and Klein, 1998; Alberts et

al, 2002). Turgor is expensive to maintain. Water and solutes must be moved against the concentration gradient. This requires energy expenditure. This would indicate that there are some advantages in the maintenance of turgor. These benefits are evident in the fact that when the hydrostatic pressure is reduced below a certain threshold, the ability to form important reproductive structures is lost along with the ability invade solid substrates. Taking the latter fact into account, it was proposed that invasive growth is predominantly driven by turgor pressure and non-invasive growth is not.

It was demonstrated during the course of this thesis that the turgor pressures of the invasive and non-invasive hyphae of *Achlya bisexualis* do not differ. This is not surprising considering this organism is incapable of turgor regulation in response to hyperosmotic shock. Mycelial morphology was shown to be dependant on the agar concentration and can be attributed to a response at the cellular level. As the force required for invasive growth is greater than that required for non-invasive growth increased turgor cannot account for the required increase in penetrative force, therefore cell wall or cytoskeletal changes must occur.

Actin is a cytoskeletal protein thought to play an essential role in the tip growth of hyphae. It has been hypothesised that this protein is integrally involved with both mechanisms of tip growth. The actin cytoskeletal network has traditionally been observed in the tips of hyphae as a fibrillar actin cap with a subapical array of plaques and filaments. Through the development of improved fixing techniques an additional structure has been resolved. This appears as an actin deplete zone at the very apex of the hyphal cells of some species. Prior to this thesis the role and the reason for the occurrence of this deplete zone had not been established. The fact that the ADZ was found consistently in significantly higher numbers of invasive compared to non-invasive hyphae suggests that it has a role in increasing the yielding at the tip. This yielding is further accomplished by an apparent weakening of the cell wall as evidenced by the significantly different pressure change required to burst the invasive compared to the non-invasive hyphae.

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